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(54) Title: PRODUCTION OF RECOMBINANT HUMAN LYSOSOMAL ALPHA-MANNOSIDASE

(57) Abstract: The present invention relates to a cell capable of producing recombinant human LAMAN (rhLAMAN), said cell comprising the 3066 basepair EcoRI - XbaI fragment of a human cDNA which codes for a human LAMAN protein in which the position corresponding to position 186 of the full length hLAMAN protein is Aspartic acid (Asp, D). In particular, the invention relates to a cell comprising a DNA sequence which codes for the amino acid sequence shown in SEQ ID NO. 12. One embodiment is an expression plasmid pLamanExp1 having the sequence shown in SEQ ID NO. 2. One aspect of the invention relates to a method for the preparation of recombinant human LAMAN, the method comprising a) introducing, into a suitable vector, a nucleic acid fragment comprising a DNA fragment which codes for the amino acid sequence shown in SEQ ID NO. 12, b) transforming a cell with the vector obtained in step a), c) culturing the transformed host cell under conditions facilitating expression of the nucleic acid sequence, d) recovering the expression product from the culture. The method may further comprise a fermentation step and/or a purification step. The invention further relates to a rhLAMAN produced by the method of the invention and to the use of the rhLAMAN produced according to the invention for the preparation of a medicament for the treatment of alfa mannosidosis.

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Production of recombinant human lysosomal alpha-mannosidase

The present invention relates to a cell capable of producing recombinant human LAMAN (rhLAMAN), said cell comprising the 3066 basepair EcoRI - XbaI fragment of a human
5   cDNA which codes for a human LAMAN protein in which the position corresponding to position 186 of the full length hLAMAN protein is Aspartic acid (Asp, D). In particular, the method relates to a cell comprising a DNA sequence which codes for the amino acid sequence shown in SEQ ID NO 12.

10 In a further aspect, the invention relates to a method for the preparation of recombinant human LAMAN, the method comprising a) introducing, into a suitable vector, a nucleic acid fragment comprising a DNA fragment which codes for the amino acid sequence shown SEQ ID NO 12; b) transforming a cell with the vector obtained in step a); c) culturing the transformed host cell under conditions facilitating expression of the nucleic acid sequence;
15 d) recovering the expression product from the culture. The method may further comprise a fermentation step and/or a purification step.

Another embodiment of the present invention relates to a method for preventing or treating the development of symptoms related to alpha-mannosidosis caused by a de-
20 ficiency, in a patient, of the human lysosomal alpha-mannosidase (hLAMAN) enzyme, the method comprising administering over cellular membranes, to a target cell, an effective amount of recombinant human Lysosomal alpha-mannosidase (rhLAMAN) enzyme.

BACKGROUND OF THE INVENTION
25

Alpha-mannosidosis

Alpha-mannosidosis is a recessive autosomal disease that occur world wide with a frequency of between 1/1.000.000 and 1/500.000. Mannosidosis is found in all ethnic
30 groups in Europe, America, Africa and also Asia. It is detected in all countries with a good diagnostic service for lysosomal storage disorders, at a similar frequency. They are born apparently healthy, however the symptoms of the diseases are progressive. Alpha-mannosidosis displays clinical heterogeneity, ranging from very serious to very mild forms. Typical clinical symptoms are: mental retardation, skeletal changes, impaired immune
35 system resulting in recurrent infections, hearing impairment and often the disease is associated with a typical facial characteristics such as a coarse face, a prominent forehead, a flattened nasal bridge, a small nose, and a broad mouth. In the most severe cases (mannosidosis type I) the children suffer from hepatosplenomegaly, and they die during

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the first years of life. Possibly this early death is caused by severe infections due to the immunodeficiency caused by the disease. In milder cases (mannosidosis type 2) the patients usually reach adult age. The skeletal weaknesses of the result in the needs of wheeling chairs at age 20 to 40. The disease causes a diffuse dysfunction of the brain

5 often resulting in weak mental performances that excludes anything but the most basic skills of simple reading and writing. These problems associated with hearing inabilities and other clinical manifestations preclude the patient from an independent life, the consequence being that life long caretaking is needed.

10 <u>Lysosomal alpha-mannosidase</u>
Alpha-mannosidosis results from a deficient activity of lysosomal alpha-mannosidase (LAMAN, EC3.2.1.24). The disease is characterised by massive intracellular accumulation of mannose-rich oligosaccharides. LAMAN is an exoglycosidase which hydrolyses terminal, non-reducing alpha-D-mannose residues in alpha-D-mannosides from the non-reducing

15 end during the ordered degradation of the N-linked glycoproteins (Aronson and Kuranda FASEB J 3:2615–2622. 1989). The human enzyme is synthesised as a single polypeptide of 1011 amino acids with a putative signal peptide of 49 residues that is processed into three main glycopeptides of 15, 42, and 70 kD. (Nilssen et al. Hum.Mol.Genet. 6, 717-726. 1997).

20

<u>The lysosomal alpha-mannosidase gene</u>
The gene coding for LAMAN (MANB) is located at chromosome 19 (19cen-q12), (Kaneda et al. Chromosoma 95:8–12. 1987). MANB consists of 24 exons, spanning 21.5 kb (GenBank accession numbers U60885–U60899; Riise et al. Genomics 42:200–207 . 1997). The

25 LAMAN transcript is » 3,500 nucleotides (nts) and contains an open reading frame encoding 1,011 amino acids (GenBank U60266.1).

The cloning and sequencing of the human cDNA encoding LAMAN has been published in three papers (Nilssen et al. Hum.Mol.Genet. 6, 717-726. 1997; Liao et al. J.Biol.Chem.

30 271, 28348-28358. 1996; Nebes et al. Biochem.Biophys.Res.Commun. 200, 239-245. 1994). Curiously, the three sequences are not identical. When compared to the sequence of Nilssen et al (accession # U60266.1) a TA to AT change at positions 1670 and 1671 resulting in a valine to aspartic acid substitution was found by Liao et al. and Nebes et al.

35 <u>Mutations</u>
In 1997 Nilssen et al. (Hum Mol Genet 6:717–726) reported the first disease causing mutation in the fibroblasts from two siblings with mannosidosis in a highly inbred Palestinian family. In these two patients, the cause of the disease is a point mutation at the gene, with the nucleotide Adenine substituted with Thymidin. Since then, several

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genetic causes to the disease have been found in man. Today mutations causing alpha-
mannosidosis have been characterised in more than 60 patients, manly in Europeans.
These mutations vary both In type, encompassing nucleotide substitutions, deletions and
additions and spanning most of the 24 exons of the gene (Berg et al. Am J Genet 64: 77-
5 88. 1999).

The consequences of the mutations on the protein level also vary, from amino acid
substitutions that effect both the active site and the folding, to early frame shifts and
premature stop codons, resulting in a completely inactive product.
10

The amino acid sequence and enzyme structure in a number of different species. When
comparing species, many of the human and the bovine mutations found are located to a
highly "conserved" region of the mannosidase polypeptide for several species like man,
cattle, cat, whale, bird, cod and slime mould, indicating that these regions are very
15 important for the enzyme function.

Despite the many different types of mutations, it seems that all the mutations cause the
similarly complete loss of enzyme activity in cells. Thus, apparently, the variations of
clinical severity's cannot be explained by variations in the enzyme activity. Rather, these
20 variations are caused by other factors, being attributed to the general ability of the
organism to buffer the obnoxious effects of the accumulating oligomannosides, and by the
environments to prevent and to treat infections and other clinical symptoms In the
patients. SInce no patients appear to possess enzyme activity, alpha-mannosidosis may be
regarded as a mild disease compared to many other lysosomal storage disorders, but
25 similar to the other glycoproteinoses (asparytlglucosaminoria, fucosidosis, Schindler's
disease, beta-mannosidosis, Salla's disease).

Based on thIs one may assume that even a very small level of enzyme activity will be
sufficient for a normal phenotype within this category of diseases.
30

Diagnosis

The diagnosis of alpha-mannosidosis is currently is based on clinical evaluation, detection
35 of mannose-rich oligosaccharides In urine, and direct measurements of alpha-mannosidase
activity in various cell types, such as leukocytes, fibroblasts, and amniocytes (Chester et
al., In: Durand P, O'Brian J (eds) Genetic errors of glycoprotein metabolism. Edi-Ermes,
Milan, pp 89–120. 1982; Thomas and Beaudet . In: Scriver CR, Beaudet AL, Sly WA, Valle

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D (eds) The metabolic and molecular bases of inherited disease. Vol 5. McGraw-Hill, New York, pp 2529-2562. 1995).

Because the symptoms initially often are mild and the biochemical diagnosis is difficult, the
5 diagnosis is frequently made late in the course of the disease. It is obvious that patients and their families would benefit substantially from an early diagnosis.

<u>Animal models</u>
Also animals may develop alpha-mannosidosis.

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Alpha mannosidosis has been described in cattle (Hocking et al. Biochem J 128:69–78. 1972), cats (Walkley et al. Proc. Nat. Acad. Sci. 91: 2970-2974, 1994), and guinea pigs (Crawley et al. Pediatr Res 46: 501-509, 1999). A mouse model was recently generated by targeted disruption of the alpha-mannosidase gene (Stinchi et al. Hum Mol Genet 8: 1366-
15 72, 1999).

Like in humans alpha mannosidase seems to be caused by specific mutations in the gene coding for lysosomal alpha-mannosidase. Berg et al. (Biochem J. 328:863–870.1997) reported the purification of feline liver lysosomal alpha-mannosidase and determination of
20 its cDNA sequence. The active enzyme consists of 3 polypeptides, with molecular masses of 72, 41, and 12 kD. Similary to the human enzyme it was demonstrated that the feline enzyme is synthesized as a single-chain precursor with a putative signal peptide of 50 amino acids followed by a polypeptide chain of 957 amino acids, which is cleaved into the 3 polypeptides of the mature enzyme. The deduced amino acid sequence was 81.1% and
25 83.2% identical with the human and bovine sequences, respectively. A 4-bp deletion was identified in an affected Persian cat; the deletion resulted in a frameshift from codon 583 and premature termination at codon 645. No enzyme activity could be detected in the liver of the cat. A domestic long-haired cat expressing a milder phenotype had enzyme activity of 2% of normal; this cat did not possess the 4-bp deletion. Tollersrud et al. (Eur J
30 Biochem 246:410–419 .1997) purified the bovine kidney enzyme to homogeneity and cloned the gene. The gene was organized in 24 exons that spanned 16 kb. Based on the gene sequence they identified two mutations in cattle.

35 <u>Medical need for alpha-mannosidosis therapy</u>

The lack of effective treatment for alpha-mannosidosis is well recognised.

5

Enzyme substitution

When lysosomal storage diseases were discovered, hopes were raised that this could be treated by enzyme substitution. Enzyme replacement therapy has proven efficient in Gaucher disease. When exogenous lysosomal glucocerebrosidase is injected into the

5   patient, this enzyme is taken up by enzyme-deficient cells (Barton et al. N Engl J Med 324:1464-1470). Such uptake is regulated by certain receptors on the cell surface. Different tissue have different receptors. It is therefore necessary to change the natural enzyme to enable uptake into the tissue of interest. If properly designed, the missing enzyme could be injected regularly like the diabetic subject injects insulin. In many In vitro

10 studies with the purified active enzyme added to the media of enzyme-deficient fibroblasts, the lysosomal substrate accumulation was corrected, but in vivo treatment has been hampered through the problem of producing the sufficient quantity of enzymes, immunological problems, and that the substituted enzyme does not pass the blood-brain barrier.

15

Bone marrow transplantation

In 1996 Walkley et al. (Proc. Nat. Acad. Sci. 91: 2970-2974, 1994) published a paper on 3 kittens with mannosisdosis that were treated with bone marrow transplantation (BMT) in 1991. In the 2 animals that were sacrificed a normalisation was seen, not only in the body,

20 but more importantly, also in brain. The 3'rd cat was well after 6 years. Normally, an   . untreated cat dies with 3-6 months. In 1987 a child with mannosidosis was treated with BMT (Will et al. Arch Dis Child 1987 Oct;62(10):1044-9). He died after 18 weeks due to procedure related complications. In brain little enzyme activity was found. This disappointing result could be explained by heavy immunosuppressive treatment before

25 death, or that it takes time for the enzyme activity to increase in brain after BMT. The donor was the mother (who as carrier must be expected to have less than 50% enzyme activity) or it may be BMT in man has no effect on enzyme function in brain.

DISCLOSURE OF THE INVENTION

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A particularly important embodiment of the present invention is a cell capable of producing recombinant human LAMAN (rhLAMAN), said cell comprising the approximately 3066 basepair EcoRI - XbaI fragment of a human cDNA which codes for a human LAMAN protein in which the position corresponding to position 186 of the full length hLAMAN protein is

35 Aspartic acid (Asp, D). In a recent paper Berg et al. (Mol Gen and Metabol 73, 18-29, 2001) described a cell capable of producing rhLAMAN. Whereas Berg et al. does not disclose the sequence of the particular cDNA used in the paper the same group has published their LAMAN sequence as GenBank accession no. U60266. In this sequence the amino acid corresponding to position 186 is Valine not Aspartic acid as in the present

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application. Interesting, Nebes et al. (Biochem.Biophys.Res.Commun. 200, 239-245.
1994) reports that the amino acid at position 186 is Aspartic acid. However, the sequence
of Nebes et al. differs from the sequence disclosed herein at many other positions. For
instance the position corresponding to position 343 in the amino acid sequence is missing
5   in Nebes et al., also the start of the protein sequence disclosed herein is absent in Nebes
et al.

A further preferred embodiment of the present invention is a cell which contains a DNA
fragment comprising the approximately 3066 basepair EcoRI - XbaI fragment of the DNA
10 fragment having the sequence shown in SEQ ID NO 2. In addition to coding for full length
hLAMAN the sequence is engineered to comprise two convenient restriction enzyme sites
for easy insertion into a wide range of suitable expression vectors.

A most preferred embodiment of the present invention is a cell obtained by use of the
15 expression plasmid pLamanExp1 since this plasmid contains signal sequences necessary to
obtain high expression levels and such a cell furthermore is suitable for amplification as
described in example 2 .

Another inventive concept of the present invention is based on the novel idea of
20 substituting the reduced hLAMAN enzymatic activity in the person having alpha-
mannosidosis simply by administering over cellular membranes, to a target cell, an
effective amount of an hLAMAN enzyme. This can thereby "assist" the enzyme that is in
deficit.

25 Accordingly, the present invention relates to a method for preventing or treating the
development of symptoms related to alpha-mannosidosis(alpha-mannosidosis) caused by a
deficiency, in a subject, of the human Lysosomal alpha-mannosidase (hLAMAN) enzyme,
the method comprising administering over cellular membranes, to a target cell, an
effective amount of an hLAMAN catalyst which is said enzyme or an enzymatically
30 equivalent part or analogue thereof.

Since mannose-rich glycoproteins accumulates in all cells of the body and causes the
symptoms of mannosidase, the target cells for hLAMAN therapy are all cells of the body.
Since the some of the most severe symptoms caused by hLAMAN deficiency are related to
35 the central nerve system (CNS) the CNS including the brain is the one important objective
of the enzyme replacement therapy. However, beneficial delivery of hLAMAN to the brain
requires that the hLAMAN are able to cross the blood-brain-barrier (BBB).

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In a preferred embodiment, the present invention involves a treatment method in which a cellular barrier such as the blood-brain-barrier (BBB) is crossed whereby the material is delivered to the target cells.

5   Effective enzyme replacement therapy of alpha-mannosidosis patients with recombinant human LAMAN (rhLAMAN) will require the uptake of an active enzyme into the target cells, and furthermore that the rhLAMAN inside the cell is transported to the lysosomes. As described this can be accomplished by tagging the rhLAMAN. The enzyme may e.g. be tagged with mannose. E.g. endothelial cells have a mannose receptor mediated protein
10   uptake, the result being that mannose tagged proteins are internalised by liver endothelial cells, but not by cells that do not have this receptor. A number of other tags are conceivable. One particular interesting type of tag is the mannose-6-phosphate (M-6-P). This tag is particular interesting since all cell-types contain this receptor, which is cirkulating between the Golgi apparatus, the endosomes and the plasma membrane.
15   Extracellular enzymes are captured by the M-6-P receptor found on the cell surface and be internalized and delivered to the lysosomes (Willingham, et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78: 6967). It is established that the secreted forms of lysosomal enzymes produced in mammalian cell lines, for example CHO cells, contain the mannose-6-phosphate tag. This should also be valid for lysosomal alpha-mannosidase, since it is
20   normally transported to the lysosomes by a mannose-6-phosphate dependent pathway.

However, enzyme replacement therapy is not effective against the acute neuronopathic variant of Gaucher disease (Prows et al. Am J Med Genet 71:16-21). To be able to deliver rhLAMAN to the brain a vehicle that can facilitate passage of the blood-brain-barrier (BBB)
25   is needed since rhLAMAN is not likely to be able to traverse over the BBB by it self. Such "vehicle" can be a polyamine-modified, a peptide-modified, an antibody-modified LAMAN or even a cell.

DEFINITIONS
30

By the term "catalyst" is herein meant either the relevant enzyme which is substituted as it is, or an enzymatically equivalent part or analogue thereof. One example of an enzymatically equivalent part of the enzyme could be a domain or sub-sequence of the enzyme which includes the necessary catalytic site to enable the domain or sub-sequence
35   to exert substantially the same enzymatic activity as the full-length enzyme. An example of an enzymatically equivalent analogue of the enzyme could be a fusion protein which includes the catalytic site of the enzyme in a functional form, but it can also be a homologous variant of the enzyme derived from another species. Also, completely

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synthetic molecules, which mimic the specific enzymatic activity of the relevant enzyme, would also constitute "enzymatic equivalent analogues".

5 By the term "hybrid molecule" is herein meant a fusion protein between the mhLAMAN catalyst and the peptide required for transport of the vehicle over the BBB and/or cellular membranes. In the said fusion protein the vehicle part can be attached to either the C-terminal or N-terminal end of the mhLAMAN catalyst.

10 By the term "recombinant techniques" is herein meant a technique which involves recombinant DNA molecules, that is hybrid DNA sequences comprising at least two DNA sequences, the first sequence not normally being found together in nature with the second.

By the terms "recombinant polynucleotide" or "recombinant polypeptide" as used herein means at least a polynucleotide or polypeptide which by virtue of its origin or manipulation
15 is not associated with all or a portion of the polynucleotide or polypeptide with which it is associated in nature and/or is linked to a polynucleotide or polypeptide other than that to which it is linked in nature.

The term "cloning vehicle" describes a plasmid, virus, retrovirus, bacteriophage, cosmid,
20 artificial chromosome (bacterial or yeast), or nucleic acid sequence which is able to replicate in a host cell, characterized by one or a small number of restriction endonuclease recognition sites at which the sequence may be cut in a predetermined fashion, and which may contain an optional marker suitable for use in the identification of transformed cells, e.g., tetracycline resistance or ampicillin resistance. A special class of cloning vectors
25 possess the features necessary for it to operate as an expression vector. An "expression vector" has a promoter positioned upstream of the site at which the sequence is cut for the insertion of the heterologous DNA sequence, the recognition site being selected so that the promoter will be operatively associated with the heterologous DNA sequence. A heterologous DNA sequence is "operatively associated" with the promoter in a cell when
30 RNA polymerase which binds the promoter sequence transcribes the coding sequence into mRNA which is then in turn translated into the protein encoded by the coding sequence.

By the term "target cell" is herein meant a cell or group of cells (tissue) to which the enzymes should be delivered.
35

DETAILED DISCLOSURE OF THE INVENTION

The present invention relates to a cell capable of producing recombinant human LAMAN (rhLAMAN), said cell comprising the 3066 basepair EcoRI - XbaI fragment of a human

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cDNA which codes for a human LAMAN protein in which the position corresponding to position 186 of the full length hLAMAN protein is Aspartic acid (Asp, D). In particular, the invention relates to a cell comprising a DNA sequence which codes for the amino acid sequence shown in SEQ ID NO 12.

5

A specific embodiment of the invention relates to a cell obtained by insertion of a DNA fragment comprising the 3066 basepair EcoRI - XbaI fragment of the DNA fragment having the sequence shown in SEQ ID NO 2, in particular a cell obtained by use of the expression plasmid pLamanExp1 having SEQ ID NO. 2. The cell according to the invention may be

10   obtained by transfection of a non-human mammalian cell line such as by transfection of chinese hamster ovary (CHO) cells.

A presently preferred embodiment of the invention relates to a cell obtainable by the culture of the human LAMAN production cell line DSM ACC2549 which has been deposited

15   at the DSMZ for the purposes of patent deposit according to the Budapest Treaty on 6 June 2002. The invention further relates to an expression plasmid pLamanExp1 having the sequence shown in SEQ ID NO. 2.

In another embodiment, the invention relates to a method for the preparation of

20   recombinant human LAMAN, the method comprising a) introducing, into a suitable vector, a nucleic acid fragment comprising a DNA fragment which codes for the amino acid sequence shown SEQ ID NO 12; b) transforming a cell with the vector obtained in step a); c) culturing the transformed host cell under conditions facilitating expression of the nucleic acid sequence; d) recovering the expression product from the culture.

25

The method may further comprise a fermentation step and/or a purification step.
In particular, the invention relates to a method wherein the nucleic acid fragment comprises the 3066 basepair EcoRI - XbaI fragment of the DNA fragment shown in SEQ ID NO 2.

30

An important aspect of the invention relates to a rhLAMAN produced by the method of the invention and to the use of the rhLAMAN according to the invention for the preparation of a medicament for the treatment of alfa mannosidosis.

35   In its broadest aspect, the present invention relates to a recombinant polypeptide and to a nucleotide sequence encoding the LAMAN polypeptide, to an expression system capable of expressing the polypeptide as well as to pharmaceutical compositions comprising the polypeptide or part of it and to the use of the polypeptide for preventing or treating the

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development of symptoms related to alpha-mannosidosis(alpha-mannosidosis) caused by a deficiency, in a subject, of the human Lysosomal alpha-mannosidase (hLAMAN) enzyme.

Preferably, the hLAMAN catalyst is administered over cellular membranes, to a target cell,
5   by uptake of hLAMAN into the target cell by taking advantage of a mannose- or M-P-6-receptor-mediated uptake.

Mannose-6-phosphate tagged hLAMAN is preferably made in a mammalian cell system (e.g. CHO, COS cells or BHK cells (Stein et al. J Biol Chem.1989, 264, 1252-1259) to
10 secure correct mannose-6-phosphate tagging on the molecule, which ensures efficient receptor mediated uptake. Mannose-6-phosphate tagged mhLAMAN is secreted into the medium and purification of rhhLAMAN may be facilitated by the use of ammonium salts (NH_4Cl) in the fermentation step.

15 Example 3 provides an example of such production of mannose-6-phosphate tagged hLAMAN

The production of recombinant hLAMAN which is mannose-6-P comprises one or several or all of the following steps (an outline):
20
A. Synthesis of rhLAMAN

Cloning of specific rhLAMAN

B. Transfection
25 2-10 µg rhLAMAN hybrid vector DNA is used for transfection by phosphate precipitate/ glycerol shock methodology, into mammalian cells (e.g. CHO, COS cells or BHK cells). Transfection might also be done with an electric shock methodology.

C. Expression of rhLAMAN
30 Synthesis of the active protein and the mannose-6-P modification of said protein is done during the expression in the mammalian cell system.

D. Purification of rhLAMAN

rhLAMAN is purified using a 6 step procedure - see example 3.
35
E. Test system for mannose-6-P receptor mediated uptake

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The ability of produced rhLAMAN to be active in a mannose-6-P receptor mediated uptake is tested by incubating rhLAMAN with normal fibroblasts or fibroblasts from alpha-mannosidosis patients. Uptake into cells is assayed by increased hLAMAN activity.

5 Example 3 shows an example of mannose-receptor-mediated uptake of hLAMAN into cells.

In the following different delivery techniques of LAMAN enzyme across the BBB and/or cellular membranes are described.

10 1) Peptides and proteins as vehicles for passage LAMAN to the target cells by passage over cell membranes and/or the BBB:
A number of studies in animals have shown that certain proteins and/or peptides may act as vehicles for passage of BBB. For instance proteins modified by the insulin fragment (Fukuta et al. Phaomacol Res 11: 1681-1688) or antibodies to the transferrin receptor
15 (Friden et al. Proc Natl Acad Sci USA 88: 4771-4775) can pass the blood-brain barrier. Also proteins modified by coupling to polyamines (Poduslo and Curran. J Neurochem 66: 1599-1606) have been reported to pass the blood-brain barrier.

2) Toxins as vehicles for passage LAMAN to the target cells by passage over cell
20 membranes and/or the BBB:

Different bacteria, plants and animals produce toxins. Toxins have many different targets such as the gut (enterotoxins), nerves or synapses (neurotoxins). Toxins can traverse cell membranes via receptor mediated processes and the embodiment of the present invention
25 is to use toxins as vehicles to passage rhLAMAN to the target cells over cellular membranes and/or the BBB. The preferred target cells are cells in the CNS and/or the peripheral nervous system.

A further embodiment of the present invention is that only the peptide pertaining to the
30 translocation over cellular membranes and/or the BBB of the toxin is used.

One example of a toxin used as a vehicle is a bacterial toxin such as Diphtheria Toxin (DT), from the *Corynebacterium Diptheriae*. Bacterial toxins exhibit a wide range of toxicities and they fall into groups by structure and function. The toxin binds to a target cell and
35 enters the cell via a receptor, and is reduced to separate fragments. The processed toxin can be divided into the following 3 domains: The catalytic domain (C), the receptor domain (R), and the translocation domain (T).

12

The catalytic fragment and the receptor fragment of the toxin or fragments thereof are replaced by the LAMAN. This fusion protein can traverse cellular membranes and/or the BBB and thereby deliver the LAMAN to the target cells. One example of the engineering of a hybrid molecule could be by recombinant technology

5

A. Synthesis of rhLAMAN

Cloning of specific rhLAMAN cDNA from human liver, placenta or spleen library.

B. Synthesis of DT cDNA

10 1. Cloning of Diphtheria Toxin cDNA.

2. Removal of the cDNA coding for the catalytic domain and the receptor domain or fragments thereof. The remaining fragment of DT is in the following termed "DT(T).

C. Construction of the DT(T)-rhLAMAN hybrid

15 1. Ligation of rhLAMAN cDNA with the DT(T) fragment.

D. Expression

1. Ligate the DT(T)-rhLAMAN construct in a vector for efficient expression in a mammalian cell system e.g. COS, BHK or CHO.

20

2. Conversion of the rLAMAN to mannose-6-P tagged protein occur during the expression in the mammalian cell system.

Other examples of bacterial toxins to be used as vehicles are Clostridium Botulinum,
25 Pseudomonas Exotoxin A produced by *Psedomonas aeruginosa*, Cholera Toxin produced by *Vibrio cholerae*, and Pertussis Toxin produced by *Bordetella pertussis*.

Further examples of toxins used as vehicles are plant toxins selected from the list of the following plant toxins: cholinesterase inhibitors, protease inhibitors, amylase inhibitors,
30 tannins, cyanogenic glycosides, goitrogens, lectin proteins, and lathyrogens, pyrrozidine alkaloids.

Yet further examples of toxins used as vehicle are toxins from shellfish (saxitoxin) and snakes (alpha-bungarotoxin). The skilled person may add further examples in light of the
35 details and characteristics of the invention.

13

3) Proteins and/or peptides isolated from bacteria or viruses as vehicles for passage rhLAMAN to the target cells by passage over cell membranes and/or the BBB:

5   The transacting element and/or transacting protein from bacteria or viruses can be used together with rhLAMAN to cross the BBB and/or cellular membranes.

Examples of bacteria are selected from the following list: Ns. meningitidis, S. pneumoniae, Hemophilus influenzae, Staphylococcus species, Proteus species, Pseudomonas species, E. coli, Listeria monocytogenes, M. Tuberculosis, Neurolues, Spirochetes Borrelia burgdorferi

10 from Iodex ricinus.

Examples of viruses are selected from the following list of virus families: Parvoviridae, Papovaviridae, Adenoviridae, Herpesviridae, Poxviridae, Picornaviridae, Reoviridae, Togaviridae, Arenaviridae, Coronaviridae, Retroviridae, Bunyaviridae, Orthomyxoviridae,

15 Paramyxoviridae, and Rhabdoviridae. Relevante examples of viruses selected from the list mentioned above are e.g. Measles virus, Papova virus, and JC virus.

4) Mannose-receptor mediated uptake of hLAMAN into cells:
Several human cells (monocytes, fibroblasts, lymphocytes) have shown to be able to cross

20 the BBB by it-self (Hickey WF, Kimura H. Science. 1988, 239, 290-292, Hickey WF et al. J Neurosci Res. 1991, 28, 254-260). These cells can be "loaded" with rhLAMAN and can act as a vehicle for transport of rhLAMAN to the brain. Preferably, lymphocytes are used as vehicles, because they have a long half-life (2-3 months). Uptake of rhLAMAN into lymphocytes will take advantage of a mannose-receptor-mediated uptake.

25

Mannose-6-phosphate tagged rhLAMAN is made in a mammalian cell system (e.g. CHO, COS cells or BHK cells (Stein et al. J Biol Chem.1989, 264, 1252-1259) to secure correct mannose-6-phosphate tagging on the molecule, which ensures efficient receptor mediated uptake. Mannose-6-phosphate tagged rhLAMAN is secreted into the medium and

30 purification of rhLAMAN may be facilitated by the use of ammonium salts (NH_4Cl) in the fermentation step.

The disease which is the target for the inventive method is alpha-mannosidosis, and therefore the catalyst is hLAMAN or an enzymatically equivalent part or analogue thereof.

35 It is most preferred that the catalyst is a recombinant form of the human hLAMAN enzyme or of the enzymatically equivalent part or analogue thereof, since recombinant production will allow large-scale production which, with the present means available, does not seem feasible if the enzyme would have to be purified from a native source.

14

Furthermore, use of such catalysts, as described herein, for the preparation of pharmaceutical compositions for treatment of the above-discussed diseases is also part of the invention.

5 Thus, the present invention represents an important advance in the treatment of genetic and/or acquired metabolic brain disorders of alpha-mannosidosis problems associated with prior treatment methods, including the gene therapy and bone marrow transplantation.

The present invention also relates to the new and surprising concept of use as a carrier of a peptide or protein from a structure capable of crossing a biological barrier, such as a
10 cellular barrier including the blood-brain-barrier or a specific membrane of a cell. The object of the carrier function is to deliver an enzyme to a target cell. The target cell is generally a cell wherein the enzyme activity is insufficient either due to an decrease activity of the enzyme or to a situation where an increased activity is desired.

15 In one embodiment, the invention relates to a method for increasing the content of an enzyme in a cell comprising delivery of the enzyme to the tissue relevant for the cell and/or to the cell by use of a protein or peptide capable of crossing a cellular barrier, the protein or peptide being derivable from the group of toxins, bacteria, from viral peptides, and from fragments and modifications thereof.
20

The disease alpha-mannosidosis has been explained in detail above and the present invention is of particular importance in connection with alleviating the progression of symptoms caused by the enzyme defect of hLAMAN. Accordingly, in a preferred embodiment, the invention relates to a method for preventing or treating the development
25 of symptoms related to alpha-mannosidosis comprising the administration of the enzyme recombinant human lysosomal alpha-mannosidase (rhLAMAN) to the tissue relevant for the cells and/or to the cells by use of a protein peptide capable of crossing a cellular barrier or by use of a human cell as a vehicle for delivering of the enzyme to the tissue relevant for the cells and/or to the cells.
30 Similar to the use of a proteins such as insulin or transferrin antibodies as carrier proteins, the use of a protein or peptide derivable from a toxin protein has been suggested as a carrier protein the present invention. Such toxins may be a toxin selected from plant toxins, bacterial toxins and from toxins from animals. The toxin may be modified in order to increase the desired properties of the peptide.
35

In one aspect, a protein or peptide derivable from a virus may be used. However, as virus in its nature has the cell nucleus as target organ for the infection, it is contemplated that a modification or specific fragment not having this effect may be a preferred embodiment.

15

However, the delivery of an enzyme to an enzyme deficient cell related to alpha-
mannosidosis by use of an protein virus is a further surprising aspect of the invention.

As virus in general is an inhomogeneous group with respect to affinity for different cells, it
5   is within the spirit of the present invention to select the concrete virus in accordance with
the desired target cell to which the enzyme is to be delivered.

Affection of the nervous system, especially of the central nervous system, very often plays
a significant role in the broad range of diseases caused by enzyme deficiency. Accordingly,
10 in a preferred embodiment, the carrier protein or peptide is one which is capable of
crossing the blood-brain-barrier.

This is possible by use of proteins or peptide which due to their nature bears a structure
relevant for such transduction. In an important method according to the invention, the
15 cellular barrier includes the blood-brain-barrier (BBB) and the tissue is a tissue and/or cell
of the central nervous system.

The overall idea of the present invention is to prepare a construct comprising the enzyme
in question the delivering protein/peptide. Accordingly, in one aspect the invention relates
20 to a method wherein the enzyme forms a hybrid with the protein or peptide capable of
crossing the cellular barrier.

As explained in further details in the text of the present specification the hybrid is
preferably produced by recombinant techniques. However, the construct may be produced
25 by techniques of protein synthesis generally known in the art including solid phase
synthesis. The complete hybrid or part of the hybrid may accordingly be produced
synthetically or a part or the hybrid construct may be produced by use of a genuine
protein or peptide. The enzyme part and delivery part may be linked by different
techniques known in the art.
30

Preferably, the hLAMAN is made by recombinant techniques. In a further embodiment, the
LAMAN is human (hLAMAN) and still more preferred mature human hLAMAN (mhLAMAN)
or a fragment thereof. The fragment may be modified, however the active sites of the
enzyme should be preserved.
35

In a still further aspect of the invention, cells are used as a vehicle for delivering an
enzyme, preferably hLAMAN to the target cell. The preferred human cell is selected from
human monocytes, human fibroblasts, and human lymphocytes. As explained above, it is
preferred for target cells of the central nervous system that the cell for delivering the

16

enzyme is capable of crossing the BBB for delivering the hLAMAN to the tissue and/or cells of the central nervous system.

5   In order to facilitate the delivery of the enzyme to the target cell, the hLAMAN may be transferred to a target cell by means of a mannose-receptor-mediated uptake. Such uptake may be further increased when the hLAMAN is a mannose-6-P tagged hLAMAN, preferable made by expressing hLAMAN in a mammalian cell system. Preferred mammalian cell system is selected from CHO cells, COS cells, and BHK cells.

10 In general, the target cell is a cell wherein the activity, such as hLAMAN is insufficient for the optimal function of the cell. Insufficient activity of hLAMAN may be measured by one or more of the parameters selected from monitoring incresed levels of mannose-rich oligosaccharides in urine , analysis of hLAMAN activity in material from the patient such as in leukocytes and/or in skin fibroblasts, presence of clinical symptoms or increase in rate of
15 development of clinical symptoms of alpha-mannosidosis.

A significant feature of insufficient hLAMAN activity is a cell wherein a massive intracellular accumulation of mannose-rich oligosaccharides is present. Naturally, such cell is a target cell according to the present invention. The target cell may also be cells of the nervous
20 system. In addition, target cells for delivering the enzyme also includes one or more cell types selected from human monocytes, human fibroblasts, human lymphocytes, human macrophages.

An increased activity of the hLAMAN may be used as a parameter for a treatment schedule
25 an may be measured by one or more of the parameters selected from monitoring incresed levels of mannose-rich oligosaccharides in urine , analysis of hLAMAN activity in material from the patient such as in leukocytes and/or in skin fibroblasts, presence of clinical symptoms or increase in rate of development of clinical symptoms of alpha-mannosidosis.

30 It is a very important aspect of the invention to perform the treatment of a possible enzyme defect prenatally. In a further aspect of the invention, the cellular membrane is the fetal-maternal barrier (placenta). It is also within the scope of the invention to deliver the enzyme-protein-construct directly to the fetus prenatally.

35 In a still further embodiment, the invention relates to an antibody raised against any of the constructs formed by any of the enzymes and any of the proteins and/or peptide mentioned herein. Such antibody may be used for the targeting of the construct, for inactivation of the construction including increasing the elimination of the construct from the subject or even more important for detecting or monitoring the level of intracellular

17

levels of LAMAN. The antibody may be a polyclonal antibody or a monoclonal antibody and by be produced by techniques known in the art.

Any enzyme construct disclosed above as well as the antibody thereto may be use for the
5 preparation of a medicament for a treatment in accordance with the method disclosed in the present context. Accordingly, the present invention also relates to a pharmaceutical medicament comprising an enzyme linked to a carrier system such as a protein or peptide or to a cell system as disclosed in detail above.

10 Accordingly, in one aspect, the present invention relates to a construct as well as to the use of a construct comprising an enzyme and/or a protein or peptide protein peptide capable of crossing a cellular barrier or a human cell as a vehicle for delivering of the enzyme to the tissue relevant for the cells and/or to the cells as disclosed in any of the methods mentioned and specified in the claims for the preparation of a medicament.
15

Also the antibody and use thereof the preparation of a medicament is within the scope of the invention.

In a presently preferred aspect, the present invention relates to the use of the LAMAN
20 coding sequence of figure 4 for the use in gene therapy. It is suggested that a recombinant viral or non-viral vector comprising the cDNA coding for the biologically active human LAMAN and able to infect and/or transfect and sustain expression of the biologically active human LAMAN in mammalian cells could be produced. Such constructs could be used to treat alpha-mannosidase patients.
25

In a very one important aspect the present invention the principle of "gene therapy" is developed in relation to therapy by bone marrow transplantation. Bone marrow transplantation (BMT) with cells that do produce LAMAN is known to have a curative effect on individuals (test animals) suffering from alpha-mannosidosis (Walkley et al. Proc. Nat.
30 Acad. Sci. 91: 2970-2974, 1994). BMT may even be beneficial to human patients. In 1987 a child with mannosidosis was treated with BMT (Will et al. Arch Dis Child 1987 Oct;62(10):1044-9). He died after 18 weeks due to procedure related complications. This disappointing result could be explained by heavy immunosuppressive treatment before death. This treatment was necessary because the patient received non-self bone-marrow
35 cells. One way to overcome the problem of rejection of foreign bone marrow cells could be to use the patients own cells. We suggest to transfect such stem cells with a construct comprising the LAMAN cDNA nucleic acid sequence to obtain bone marrow stem cells that express enhanched levels of LAMAN, and finally to reintroduce these cells into the bone marrow of the patient.

18

5

LEGEND TO FIGURES

Figure 1.

10 Description of oligonucleotides used for PCR amplification.
    The two oligonucleotides ICO1106 and ICO1107 are shown below the LAMAN sequence.
    Numbering refers to the numbering in the GenBank sequence Accession # U60266.1. The
    highlighted regions in the LAMAN sequence represent the regions of homology with the
    oligonucleotide primers. The asterisk denotes the C to A change introduced at position 53
15 to generate a *Xho* I site for ease of manipulation. This is a silent mutation in the codon for
    the arginine residue at position 13 in the amino acid sequence of the α-mannosidase
    protein. The triplets coding for this arginine residue and for the termination codon are
    boxed. The PCR primer ICO1107 has a 5' extension incorporating a *Xba* I site to allow
    cloning of the amplified DNA.
20
    Figure 2.
    Strategy for PCR cloning of LAMAN.

    Figure 3.
25 Map of the expression plasmid pLamanExp1.
    "pCMV" Is the Human cytomegalovirus Immediate-early promoter/enhancer. "Intron" is a
    chimeric intron. "rhLAMAN" is the recombinant human lysosomal alpha monnosidase
    coding region. "SV40-term" is the SV40 late termination and polyadenylation signal. "F1'"
    Is Phage f1 region. "SV40 Eprom" is the SV40 early promoter/enhancer and origin. "DHFR"
30 Is the Mouse dihidrofolate reductase coding region. "pA" Is a synthetic polyadenylation
    signal. "AmpR" Is the beta-lactamase (AmpR) coding region.

    Figure 4.
    Map of the expression plasmid pAsaExp1.
35 "pCMV" Is the Human cytomegalovirus immediate-early promoter/enhancer. "Intron" Is a
    chimeric intron. "rhASA" is the recombinant human Arylsulfatase A coding region coding
    region. "SV40-term" Is the SV40 late termination and polyadenylation signal. "F1'" is
    Phage f1 region. "SV40 Eprom" is the SV40 early promoter/enhancer and origin. "DHFR" Is

19

the Mouse dihidrofolate reductase coding region. "pA" is a synthetic polyadenylation signal. "AmpR" is the beta-lactamase (AmpR) coding region.

Figure 5. SDS-PAGE analysis of anion exchange chromatography for rhLAMAN. See table 2
5   for content of each lane.

Figure 6.   SDS-PAGE analysis of anion exchange chromatography for rhLAMAN (larger scale). See table 3 for content of each lane.

10   Figure 7. Fibroblasts from a patient with mannosidosis (GM00654) loaded with DEAE-purified rhLAMAN for 40 hours in complete medium.

Figure 8. Fibroblasts from a patient with mannosidosis (GM00654) loaded with DEAE-purified rhLAMAN for 40 hours in complete medium with the addition of 5 mM Mannose-6-
15   Phosphate (M-6-P) or 5 mM Glucose-6-Phosphate (G-6-P).

Figure 9. Fibroblasts from a patient with alpha-mannosidosis (GM00654) loaded with DEAE-purified rhLAMAN for 40 hours in complete medium with the addition of 5 mM Mannose-6-Phosphate (M-6-P) or 5 mM Glucose-6-Phosphate (G-6-P). Endogenous LAMAN
20   activities are subtracted from each bar.

Figure 10. Fibroblasts from a patient with alpha-mannosidosis (GM00654) loaded with DEAE-purified rhLAMAN for 40 hours in complete medium with the addition of 5 mM Mannose-6-Phosphate (M-6-P) or 5 mM Glucose-6-Phosphate (G-6-P).
25
Figure 11. Fibroblasts from a patient with alpha-mannosidosis (GM00654) loaded with DEAE-purified rhLAMAN for 40 hours in complete medium with the addition of 5 mM Mannose-6-Phosphate (M-6-P) or 5 mM Glucose-6-Phosphate (G-6-P) with the endogenous LAMAN activities subtracted from each bar.
30
EXAMPLES

Example 1: Cloning of hLAMAN cDNA and construction of expression vector

35   PCR and cloning
The source of the PCR DNA template was a HepG2 cDNA library made from mRNA isolated from the HepG2 cell line (ATCC # HB-8065) using the Superscript Plasmid System manufactured by Gibco BRL, catalogue # 18248-013. Two hundred ng of DNA from this library was amplified using Klentaq polymerase mix (Clontech, catalogue # 84171-1) In a

20

50 ul reaction volume containing 0.2mM dNTPs and 0.4 uM each of ICO1106 (5' TCTGCGC TCGAGGCTGCCTGGACTCA, SEQ ID NO. 3) and ICO1107 (5' TGCTAATCTAGAGGG CCCATCCCAGCAGAC, SEQ ID NO. 4). Two-cycle PCR was used with an initial denaturation step at 96°C for 2 minutes followed by 30 cycles of 94°C for 30 seconds and 65°C for 4.5
5 minutes. A final extension of 10 minutes at 72°C was used at the end to ensure that the extension products were filled out.

The PCR reaction mix was run out on a gel and a PCR product of 3038 bp was isolated. One twentieth of this material was re-amplified with the same two primers as described
10 above, except that the temperature of the extension reaction was 68°C. The 3038 bp amplified fragment was gel isolated, digested with *Xho* I and *Xba* I and assembled into plasmid pAsaExp1 (Fig 5 and 6) cut with *EcoR* I and *Xba* I together with oligos ICO1108 (5'AATTCGCCGCCATGGGCGCCTACGCGCGGGCTTCGGGGGTCTGCGC) and ICO1109 (5'TCGAGCGCAGACCCCCGAAGCCCG CGCGTAGGCGCCCATGGCGGCG) in a three part
15 litigation (Fig.2).

Sequencing
Seven plasmid clones from the ligations described above were sequenced with the Big dye terminator cycle sequencing kit from PE/ABI (catalogue # 4314417). Two vector primers,
20 ICO873 (5' TGCAGCTTATAATGGTTACA, SEQ ID NO. 5) and ICO929 (5' GGCACCTATTGGTCTTACTG, SEQ ID NO. 6) and five LAMAN-specific primers, ICO1110 (5' AAGTCGTGCGAGACCTTGTG, SEQ ID NO. 7), ICO1111 (5' CCGCCAAGGA ATCTGTGCTG, SEQ ID NO. 8), ICO1112 (5' TACGAGCGCCTCAGCTACAA, SEQ ID NO. 9) and ICO1113 (5' GCGGAAGGTGAATTGGATGG, SEQ ID NO. 10) and ICO1114 (5' GCCTCAGGTGCC
25 TACATCTT, SEQ ID NO. 11) were used. The results are summarized in Table 1.

Construction of the correct, full-length LAMAN cDNA
As seen from Table 1, none of the seven cDNA clones match the published sequence (Nilssen et al. Hum.Mol.Genet. 6, 717-726 1997, revised version Accession # U60266.1).
30 In addition to a number of random changes they all have the TA to AT changes at positions 1670 and 1671 in the plasmid sequence (SEQ ID NO:1). This results in a valine to asparatic acid substitution in residue 186 of the LAMAN protein sequence, see SEQ ID NO. 12. The same valine to asparatic acid change is also reported by two other research groups (Liao et al. J.Biol.Chem. 271, 28348-28358, 1996 and Nebes et al. Biochem
35 Biophys. Res. Commun. 200, 239-245, 1994). Hence, for the purpose of this study, a full-length LAMAN sequence was patched together from plasmid clones pLaman-50 and pLaman-70 using the unique SanD I site at position 2388 (Figs. 2 and SEQ ID NO:2).

A 1287 bp EcoR I – SanD I fragment from pLaman-50 and a 1779 bp

21

SanD I – Xba I fragment from pLaman-70 were isolated and assembled together in
plasmid pAsaExp1 (Fig. 5 and 6) cut with EcoR I and Xba I in a three part ligation (Fig. 2).
The resulting plasmid is called pLamanExp1. The LAMAN insert in this plasmid was
confirmed by sequencing and found to match the sequence (Nilssen et al. Hum.Mol.Genet.
5 6, 717-726, 1997 accession # U60266.1) except at amino acid position 186 as referred to
in the plasmid sequence shown in SEQ ID NO:2. The map of this plasmid is shown in Fig.
3.

22

Table 1. Summary table of sequencing results

Clone #	Nucleotide change	Amino acid change
27	1287, G to A	58, V silent
	1292, C to T	60, P to L
	1320, T to G	69, P silent
	1670, T to A	186, V to D
	1671, A to T	186, V to D
	2048, G to T	312, T to
	2829, C to T	572, A silent
	3423, C to T	770, P silent
	3448, G to A	779, N to D
	3826, C to T	905, L silent
	3835, T to C	908, W to R
	4112, T to C	1000, F to S
46	1258, G to A	49, A to T
	1670, T to A	186, V to D
	1671, A to T	186, V to D
	2360, A to G	416, E to G
	2415, A to G	434, A silent
	3409, A to G	766, N to D
50	1670, T to A	186, V to D
	1671, A to T	186, V to D
	2401, T to C	430, S to P
	2465, G to A	451, G to D
	2824, C to T	571, P to S
	2886, C to T	591, R silent
	3075, C to T	654, D silent
	3281, A to G	723, D to G
	3423, C to T	770, P silent
	3488, C to T	792, T to I
66	1165, T to A	18, S to T
	1670, T to A	186, V to D
	1671, A to T	186, V to D
	2450, A to G	446, H to R
	2686, A to G	525, M to V

23

	3368, T to C	752, I to T
	3423, C to T	770, P silent
70	1391, A to G	93, Q to R
	1670, T to A	186, V to D
	1671, A to T	186, V to D
	1869, C to T	252, L silent
	1976, A to G	288, N to S
	2048, C to T	312, T to I
	2146, A to G	345, K to E
	3186, G to A	691, Q silent
	3423, C to T	770, P silent
74	1670, T to A	186, V to D
	1671, A to T	186, V to D
	1823, A to G	237, Q to R
	1849, A to G	246, K to E
	1910, A to G	266, N to S
	2012, C to T	300, A to V
	2048, C to T	312, T to I
	2284, T to G	391, Y to D
	3330, A to G	739, T silent
	3558, A to T	815, R silent
	3801, G to T	896, L silent
	3813, G to A	900, V silent
	3828, G to A	905, L silent
76	1169, C to T	19, A to V
	1670, T to A	186, V to D
	1671, A to T	186, V to D
	2653, G to A	514, V to D
	3008, A to G	632, Q to R
	3724, C to T	871, L silent

NOTE: The clones are in plasmid pLamanExp1. The numbering is as shown in the plasmid sequence (SEQ ID NO:2). Identical changes between clones are highlighted in different
5   colors. The reference sequence for the lysosomal alpha-mannosidase cDNA has the GenBank Accession number U60266.1 (1, revised).

24

Discussion

The inserts in all seven of the clones were confirmed to be LAMAN by sequence analysis. As seen in Table 1 a number of changes were found in all of the clones when compared to

5   our reference sequence (1, revised version accession # U60266.1). Most of them are likely to be random PCR errors. Curiously, the TA to AT change at positions 1670 and 1671 resulting in a valine to asparatic acid substitution was common to all the clones. The two other sequences published for LAMAN (Nebs et al. Biochem. Biophys. Res. Commun. 200, 239-245, 1994, Liao et al. J.Biol.Chem. 271, 28348-28358, 1996) also report asparatic

10   acid in that position. In the light of these observations a full-length correct sequence was put together with the help of the SanD I site at position 2388 as described under materials and methods. The sequence of the cDNA insert in this clone is identical to the reference sequence except for the valine to asparatic acid change mentioned above.

15   Evaluation and conclusions

A combination of PCR amplification and incorporation of synthetic oligonucleotides assembled a full-length clone of LAMAN. It matches the sequence Accession # U2066.1, our reference sequence except in the valine to asparatic acid substitution alluded to earlier   . in this report. This LAMAN cDNA sequence also carries a C to A change at nucleotide

20   position 1152 (see plasmid sequence in SEQ ID NO. 2). This was engineered to create a Xho I site for ease of manipulation and does not change the primary sequence of the LAMAN protein. It also contains a G to A change at position 3186 and a C to T change at   ᵢ position 3423 with no effect on the primary amino acid sequence of the LAMAN protein.

25   All seven of our clones had the valine to asparatic acid substitution in the 186th residue of the protein. Since the same substitution was also reported by two other groups (accession #s U68567 and U05572, refs. 2 and 3) the asparatic acid residue was left unchanged at that position. The reference sequence Accession # U60266.1 differs from the two other published sequences mentioned above at a few other positions. When compared to

30   sequence Accession # U68567 (Liao et al. J.Biol.Chem. 271, 28348-28358, 1996) three other changes can be found. Curiously, the threonine to isoleucine change occurring at amino acid position 312 (Liao et al. J.Biol.Chem. 271, 28348-28358, 1996) was present in three of our clones (Table 1). None of our clones had either the 3 base-pairs deletion encoding the glutamine residue at position 342 or the G to T silent mutation in the leucine

35   residue at position 753 found in (3, Accession # U05572). Three of our clones however, had a C to T silent change in the proline at position 770.

25

Example 2: Transfection, selection of producer cell and preliminary characterisation of recombinant human LAMAN

5 Materials and methods:

Materials used for cell culture

Media:

Material	Company	Catalog #
Penicillin/Streptomycin	BioWhittaker	17-602E
L-Glutamine, 200 mM	BioWhittaker	17-605E
HT Supplement (100X)	Gibco BRL	11067-030
Ex-Cell 302 Serum-Free Media	JRH BioSciences	14312-78P

Media for DG44.42 Cells:

Ex-Cell 302 Serum-Free Medium	500 ml
Penicillin/Streptomycin	5 ml
L-Glutamine (4 mM final concentration)	10 ml
100X HT Supplement	5 ml

Media for ASA Masterwell Cells:

Ex-Cell 302 Serum-Free Medium	500 ml
Penicillin/Streptomycin	5 ml
L-Glutamine (4 mM final concentration)	10 ml
20 nM Methotrexate	0.5 ml (20 µM MTX*)

25

*Diluted from 20 mM Stock.

Preparation of 20 mM Methotrexate Stock:

30 Materials:

1. Methotrexate, U.S.P. : ICN catalogue # 102299
2. WFI (Water for Injection)
3. 1.0N NaOH to adjust pH.
4. 0.2µm filter unit of appropriate size

26

Gel electrophoresis:

Gel electrophoresis was done using the Novex system with Nupage Bis/Tris gels run at 200
volts for 1.2 hours. Staining was done with Coomassie brilliant blue R-250 according to the

5   manufacturer's specifications. Total protein was measured by the Bradford method using
the BioRad reagent (catalogue # 500-006) and bovine serum albumin as standard.

LAMAN enzyme assay
Materials:

10 4X assay buffer consisting of:
250 µM p-Nitrophenyl-alpha-D-mannopyranoside
100 µg/mL BSA
200 mM Na Acetate (pH 4.5)

15 1.8 M Na_2CO_3

Procedure:

For screening purposes the assays were done In flat-bottomed ELISA plates. 50 µl of the
4X assay buffer was added to150 µl of sample or an appropriate dilution of it (in 10 mM

20 Tris pH 7.4 containing 150 mM NaCl). The plates were incubated overnight at room
temperature, stopped with 100 µl of 1.8 M Na_2CO_3 and the absorbance recorded at 405 nm
on a plate reader.

For determination of specific activity of the DEAE-purified samples, the assays were set up

25 In tubes with all the volumes doubled. Incubations were at 37°C for periods ranging from
3-60 minutes using 10-10,000 ng of enzyme. The samples were read on a
spectrophotometer using a cuvette of 1 cm path length. Specific activity is defined as
µmoles of p-Nitrophenyl-alpha-D-mannopyranoside hydrolysed per minute per mg protein.

30

Introduction

To be able to produce rhLAMAN the expression plasmid (pLamanExp1, Fig 3, SEQ ID NO.
2) was cell type the following experiment was performed.
To obtain the final clonal cell line expressing rhLAMAN, the expression plasmid

35 pLamanExp1 (Fig 3, SEQ ID NO. 2) was transfected into the host cell line DG44.42
(obtained from DG44 by limiting dilution). High LAMAN producing masterwells, as
determined by LAMAN activity, were selected.

27

To further increase the yield, the cells from high LAMAN producing masterwells were subjected to amplification with methotrexate (MTX) as described by Gasser et al. Proc. Natl. Acad. Scl. U.S.A 79, 6522-6526. 1982. After amplification, the best producers were cloned twice by limiting dilution.

5

Transfection conditions and selection of high producing masterwells

1×10^7 CHO DG44.42 cells were resuspended in 800 μl Hepes Buffered Electroporation Media with 1020 μg pLamanExp1 DNA (linearized with *Ahd* I) +120 μg salmon sperm DNA in a 0.4 cm electroporation cuvette. The cells were electroporated using a Bio-Rad Gene

10 Pulser II set at 250 volts with 500 μFd capacitance. The contents of the cuvette were then transferred to a T-75 flask containing 20ml of JRH Ex-Cell 302 Serum–Free Medium + HT (hypoxanthine and thymidine) + 4 mM L-glutamine + 100 units/ml penicillin + 100 μg/ml streptomycin and then placed in a 37 °C, 5% CO_2 incubator. 24 hours later the cells were counted, centrifuged and transferred to two T-flasks, one with 20 nM and the other with 50

15 nM MTX replacing the HT in the growth media described above. A media sample was saved and run in a LAMAN activity assay - see materials and methods. The culture showed activity and was plated in 96-well dishes as follows; 2 plates each at 500 and 1000 cells/well in 100 nM MTX and 5 plates each at 50, 100 and 200 cells/well in 20 and 50 nM MTX respectively. The wells were fed fresh media every 5-7 days. Four weeks post

20 electroporation a LAMAN activity assay was done on media from wells containing 1 to 4 clones. The masterwells with the highest LAMAN activity were then expanded and run in a 6-day LAMAN activity assay. In a 6-day LAMAN assay cells were seeded at 1×10^5 cells/ml in 5 ml media with MTX in a T-12.5 flask. Six days later the cells were counted. The cells were then centrifuged and the supernatant analyzed in the LAMAN activity assay. Results

25 were normalized for cell number. The 12 best masterwells were then amplified.

Amplification

Six masterwells were amplified starting from 20 nM MTX and six starting from 50 nM MTX. Amplification was done by seeding cells at 1×10^5 cells/ml in T-25 Falcon flasks, in 10ml of

30 media. The cells were split every 4-6 days and analyzed in a 6-day assay. The cells were then amplified to the next level (50, 100, 200 nM MTX) on the next split as well as the cells being maintained at previous MTX levels. No amplification occurred at 100 and 200 nM MTX. The highest producing masterwell at 50 nM MTX (20L-31) was selected for further subcloning.

35

1st Round Limiting Dilution Cloning

20L-31 was spinner adapted before cloning. The cells were seeded at 1.25, 2.5, 5 and 10 cells/well in 100 μl media containing 10% conditioned media in a flat-bottom 96-well plate. They were fed with 100 μl fresh media every 5-7 days. The wells were scanned for single

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clones within 4 weeks and these cells were transferred to 24-well plates with 1 ml fresh media. Growing clones they were transferred to a T-25 flasks, expanded and run in the 6-day LAMAN assay. Twenty-seven clones were analyzed in this fashion. One clone was chosen on the basis of cell growth, viability and activity. The clone chosen, 20L-31.62, had
5   twice the LAMAN activity as the corresponding masterwell.

2nd Round Limiting Dilution Cloning

The first round clone 20L-31.62 was already spinner adapted and was taken through the same subsequent steps of limiting dilution cloning as described above. Twenty-seven
10   clones were analyzed in a 6-day LAMAN assay and one clone was retained as the final clone on the basis of cell growth, viability and LAMAN activity. The clone chosen, 20L-31.62.5, was also twice as active as the masterwell. It was maintained in media containing 50 nM MTX. This clone has been designated "CHO-LAMAN 20L-31.62.5 020118".

15

Characterization of rhLAMAN

The characterization of rhLAMAN for specific LAMAN activity was done with partially purified material from the first round clones.

20

Purification

A preliminary purification of rhLAMAN was attempted for the purposes of determining specific activity and characterise the product by gelelectrophoresis.

25   Briefly, 800 ml of conditioned media from the masterwell, 20L-31 (cultured for in T-flasks for 10 days) was dialyzed against 20 mM Tris, pH 7.6 and loaded on a 5 ml DEAE-Sepharose column (AP-Biotech, Catalogue # 17-0709-01), equilibrated in the same buffer. The column was washed thoroughly with equilibration buffer containing 20 mM NaCl and then eluted in steps using 120 mM, 250 mM and 1 M NaCl. One ml fractions were collected
30   and analyzed by Bradford protein assay and SDS-PAGE. The gel profile is shown in Fig. 5. The fractions are labeled serially according to the salt concentration used for elution. Two pools were collected. The content of each lane i described in table 2 below.

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Table 2: Contents of 4-12% BisTris w MES/SDS reducing gels

Lane	Sample	µg/ml by Bradford	total µg in lane		Lane	Sample	µg/ml by Bradford	total µg in lane
1	Marker				13	250.5	460	9.2
2	Start	124	2.48		14	marker		
3	Flow Through	80	1.6		15	1.1	1023	20.46
4	120.6	1827	36.54		16	1.2	ND	ND
5	120.7	1755	35.1		17	1.3	ND	ND
6	120.8	1250	25		18	1.4	ND	ND
7	120.9	963	19.26		19	1.5	ND	ND
8	120.10	619	12.38		20	1.6	1471	29.42
9	250.1	477	9.54		21	1.7	ND	ND
10	250.2	361	7.22		22	1.8	ND	ND
11	250.3	303	6.06		23	1.9	ND	ND
12	250.4	334	6.68		24	1.10	ND	ND

The samples correspond to the fractions collected. The fractions are

numbered serially according to the salt concentration in the step gradient used

5   (120 mM, 250 mM, 1 M). ND stands for not done.

The 120 mM pool (lanes 4-8 in Fig. 5) had a specific activity of 0.8 U/mg and 1.25 mg/ml total protein. The 250 mM pool (lanes 9-12 in Fig. 5) had a specific acivity of 1 U/mg and 0.48 mg/ml total protein.

10   Another purification was attempted starting with 1.2 L of dialyzed medium from 20L-31 grown in T-flasks applied to a 50 ml DEAE-Sepharose column. After washing the column in the same manner, rhLAMAN was eluted with a steep linear gradient of 20-120 mM NaCl. 5 ml fractions were collected and analyzed by SDS-PAGE. Figure 6 shows the gel profile and the pooled fractions are marked. The content of each lane is described in table 3 below.

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Table 3: Contents of 4-12% BisTris w MES/SDS reducing gel

Lane	Fraction	mM NaCl
1	10 µg of rhLaman from purification shown in Fig. 5	120 mM by Step elution
2	Markers	
3	5	40
4	6	40
5	10	50
6	11	50
7	12	50
8	16	60
9	18	70
10	60	140
11	62	140
12	63	150

5   The pooled material (25 ml) had a specific activity of 2 U/mg at a protein concentration of
1 mg/ml.

Conclusion

10

A LAMAN production cell line (CHO DG44.42 subclone 20L-31.62.5) was obtained after
MTX amplification and two rounds of cloning. This clone has been designated "CHO-LAMAN
20L-31.62.5 020118" and deposited at the DSMZ (Deutsche Sammlung von
Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, 38124 Braunschweig,
15 GERMANY) for the purposes of patent deposit according to the Budapest Treaty on 6 June,
2002.

The CHO cell-based expression system described here produces in excess of 20 mg/L
rhLAMAN, which is secreted into the culture medium as a single-chain molecule. A
20 preliminary purification was attempted. The purification resulted in a partially purified
rhLAMAN preparation, see elution profile Fig 5 and 6 that can be used for cellular uptake
studies and further purification.

25

31

Example 3: In vitro characterisation of rhASA

Materials and methods:

5　Complete medium

MEM Eagle-Earles BSS with
15% FCS
2 mM Glutamine
2X conc. Vitamins
10　2X conc. non-essential AA
2X conc. MEM-AA
1 mM Na-puruvate

TBS composition

15　10 mM Tris-HCl
150 mM NaCl
Adjust pH to 7.5

Alfa-Mannosidase activity assay protocol

20

4x assay buffer
16 mM para-Nitrophenyl-alpha-D-mannopyranoside
2 mg/ml BSA
in 0.4 M NaAc pH 4.5

25

Stop buffer

1.8 M Na_2CO_3

For measuring activity in cell supernatants, the assay was run in microtiter plate. For cell
30　lysates, the assay was performed in Eppendorf tubes, see below.

Microtiter plate set-up
150 μl of sample at appropriate dilution (in TBS)
50 μl of 4x assay buffer

32

The plate is incubated at 37°C for 30 minutes.
Stop by adding 100 μl stop buffer and read the plate at OD 405.

Eppendorf tube set-up

5   300 μl of sample at appropriate dilution (in TBS)

100 μl of 4 x assay buffer

The tubes are incubated at 37°C for 30 minutes to 2 hours.

Stop by adding 200 μl stop buffer, centrifuge the tubes (13 200 rpm, 10 minutes) and

transfer the supernatant to a cuvette.

10   Measure at OD 405.

The LAMAN activity is then calculated using the millimolar extinction coefficient for the
product in the formula:

15   $(OD\ 405 \times V)/(18.8 \times t \times v) = U/ml$

V = total volume for assay (ml)

t = incubation time (min)

v = sample volume (ml)

20   The millimolar extinction coefficient for para-nitrophenol = $18.8\ M^{-1}\ cm^{-1}$

1 U of LAMAN activity is defined as the amount of enzyme that liberates 1 μmol para-
nitrophenol/min, 37°C, pH 4.5.

25   <u>BCA protein determination kit</u>

Protein concentration was determined using the BCA Protein assay kit (no. 23225) from
Pierce. This kit utilizes the principle of the reduction of Cu^{2+} to Cu^{+} by protein in an alkaline
medium (the Biuret reaction). The Cu^{+} ions are then reacted with a reagent containing
bicinchoninic acid resulting in a highly sensitive and selective colorimetric detection. The
30   kit was performed according to the manual.

Microtiter plate set-up

10 μl of the BCA-Standards for a standard curve

35   10 μl of BSA reference in duplicate

10 μl of MilliQ water used as blank in at least triplicate

10 μl of sample or diluted sample in MilliQ water

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200 µl of BCA assay buffer in all wells

Mix ~30 seconds

Incubate samples for 30 minutes at 37° C

Measure the absorbance within 10 minutes at 562 nm and determine the concentrations
5   from the standard curve.

Introduction

At an early stage, before the purification procedure was developed, the rhLAMAN was
10 characterized in an *in vitro* cell system. For this purpose, LAMAN-containing supernatants
from LAMAN-CHO cells were purified using a DEAE purification step. Briefly, LAMAN-
containing supernatants from T flasks with the DG44.42 subclone 20L-31.62.5 were
diafiltered and concentrated and then added to the column after equilibration with 10 mM
Tric-HCL, pH 7.6. After loading, the column was washed with the same buffer and then
15 with 10 mM Tris-HCl + 0.02 M NaCl. 3 column volumes were used for each washing step.
The bound proteins were eluted using a NaCl gradient ranging from 0.02 to 0.3 M NaCl. 5
ml fractions were collected. The fraction with the highest specific activity was purified
approximately 100 times and the specific LAMAN activity was around 4 U/mg. This material
was used in the cell systems described below.
20

1. *Cellular uptake of rhLAMAN by MANN fibroblasts*

Aim of the study

To get *in vitro* proof of concept regarding cellular uptake of rhLAMAN in fibroblasts from
25 individuals affected by alpha-mannosidosis (MANN fibroblasts).

Experimental design

Fibroblasts from a patient with mannosidosis (MANN fibroblasts GM00654, purchased from
Coriell Cell Repository, USA) were grown confluent in 6-well plates in complete medium.
30 Cells were washed with PBS and rhLAMAN (diafiltrated, concentrated and DEAE-purified,
see purification part for details) was added (0-200 mU/ml) ± 5 mM Mannose-6-Phosphate
(M-6-P) or Glucose-6-Phosphate (G-6-P, control) in complete medium. The specific LAMAN
activity of the added enzyme was 4 U/mg total protein. The cells were left for 40 hours and
then harvested; medium was removed, centrifuged and buffer was changed to TBS using
35 PD10 columns. Cells were washed with PBS and trypsinated, washed with TBS and then
lysed with 0.5 % Triton X-100 in TBS. The lysed cells were centrifuged (13 200 rpm, 10
minutes) and lysate was collected. LAMAN activity and protein concentration were
measured in all samples.

34

For details on the LAMAN activity assay and complete medium composition, see Materials
and methods. Protein determination was performed in micro-titer plate according to our
internal SOP. See Materials and methods for a brief description of the method.

5

Results and discussion

The MANN fibroblasts had a background LAMAN activity of 1-1.5 mU/mg total protein.
DEAE-purified rhLAMAN gave a maximal cellular load of 37 mU/mg total protein (after
addition of approximately 75 mU/ml for 40 hours), see Fig. 7. Addition of M-6-P inhibited

10　90 % of the cellular uptake and there was no inhibition using G-6-P. This suggests that the
enzyme is taken up mainly by the M-6-P receptor. Results are shown in Fig. 8 and 9.

Example 4: Small-scale cultivation of producer cell

Cell line and culture medium

15　A DHFR minus Chinese Hamster Ovary (CHO) cell line, DG44.42 subclone 20L-31.62.5,
expressing rhLAMAN, is routinely cultivated in modified EX-CELL 302 CHO serum-free
medium without phenol red (JHR Biosciences Europe, UK). The medium contains 1.6 g/L
sodium bicarbonate, 4 mM HEPES and 0.1 % Pluronic®F-68, and is supplemented with 4
mM L-Glutamine and 3.4 g/L D-(+)-Glucose. The pH of the medium is 7.0-7.4. Selection is

20　maintained by using 20 nM methotrexate (MTX), a competitive inhibitor of DHFR. 1 mL of
an 20 μM MTX working solution is added to 1 L of complete medium.

No antibiotics are used. Samples for mycoplasma test are obtained periodically and
analysed by the Mycoplasma Laboratory at the National Veterinary Institute, Uppsala,

25　Sweden. All samples tested were found to be negative for mycoplasma.

T-flasks

T-flasks (175 cm^2) with 60 mL medium are seeded with 2.5-3 x 10^5 cells/mL and incubated
at 37 °C and 5 % CO_2 in a humidified incubator for 72-96 hours. After 3-4 days, a cell

30　concentration of 1-1.2 x10^6 cells/mL is reached. The T-flasks are gently tapped to break
any cell aggregates. The cell suspensions are centrifuged (110g, 8 min) in sterile tubes in
a swing-out centrifuge (Eppendorf) at 20 °C. The collected supernatants are filtered (0.45
μm) and passed on to the purification process or stored frozen (–20 °C) for later use. Fresh
and filtered supernatants from T-flasks hold a LAMAN activity of 30 mU/mL. The cell pellets

35　are resuspended in fresh medium at RT to a suitable cell density and passaged to new T-
flasks cultures.

35

Counting of viable and dead cells is performed by using a haemocytometer and the trypan blue exclusion technique. Stored cells are frozen in liquid nitrogen in a working cell bank. New vials of cells are thawed regularly, at least every fifth month, and the LAMAN production is checked every week during the cultivation process.

5

Example 5: Partially developed purification scheme, formulation, filling and lyophilization

A purification process for rhLAMAN in 20-200 ml scale is developed for scale-up to large scale production. The quality and purity of the final product (rhLAMAN) is very high and according to the specifications (approved for clinical trials). The process includes a capture

10 step, 1-2 intermediate purification steps, 1 polishing step, 1-2 virus removal steps and 1 formulation step. 1 or more buffer exchange steps are also included (dia-filtration). The small-scale process is transferred to intermediate and finally large-scale production.

Experimental design: Several different chromatography gels are tested and performance of

15 the different steps will be analysed by a battery of analytical methods described briefly below:

Enzyme activity:	Alpha-mannosidase assay
Total protein concentration:	BCA analysis
20 rhLAMAN concentration:	rhLAMAN ELISA
Purity:	HPLC and SDS-PAGE
Identity:	HPLC
HCP proteins:	ELISA
Endotoxin level:	Outsource to contract Lab

25

Outline of purification process in 20 ml column scale

Step 1: Concentration/Diafiltration

Media produced in T-500 flasks (0.06U/ml) with expressed rhLAMAN is concentrated

30 approximately 10x for using Tangential flow filtration (TFF) against a Pellicon Biomax polysulphone filter with 100 kDa cut off.

Example: 0.5 – 1.0 litre spinner produced media is concentrated using TFF against a 50 cm^2 Biomax 100 kDa filter to 50 - 100 ml. Transmembrane pressure (TMP) is 25 (Pin = 30

35 psi; Pout = 20 psi).

36

After that, diafiltration against 7 volumes of 20 mM Tris-HCl, pH 7.6 with TMP of 25 is applied. Specific activity of concentrated sample is 0.5 – 1.5 U/mg. Yield 70 – 90%

Step 2: Capture step – DEAE sepharose FF

5   Concentrated sample from step 1 is applied on a 20 ml DEAE sepharose packed in a 16 mm diameter column (Pharmacia XK 16) equilibrated with 20 mM Tris-HCl pH 7.6 (referred to as: standard buffer). Flow rate is 3 ml/min. Protein bound to the DEAE gel is then washed with 2-4 column volumes (CV) of standard buffer followed by 2-4 CV's of 30 mM NaCl in standard buffer.

10

rhLAMAN is eluted with a linear gradient from 30 – 300 mM NaCl in standard buffer for 20 minutes. Alternatively for the large scale production: Elution is achieved by applying 75 – 150 mM NaCl in standard buffer. The DEAE gel is washed with 2-4 CV's of 0.5 M NaCl in standard buffer followed by 2.4 CV's of 1.0 M NaOH (contact time 40 – 60 minutes).

15   Fractions containing rhLAMAN activity are pooled (specific activity: 2 - 5 U/mg) and used for further purification. Yield 70 – 90 %.

Step 3: Intermediate step 1

20   1.   *Hydrophobic interaction chromatography: butyl, phenyl or octyl sepharose FF.*
         Sample pool from step 2 is mixed 1:1 with 2.0 M Na_2SO_4 and applied on a 20 ml HIC column (butyl-, phenyl- or octyl-sepharose) packed in a 16 mm diameter column (Pharmacia XK 16) equilibrated with standard buffer + 1.0 M Na2SO4. Flow rate is 3-4 ml/min. Column is washed with 2-4 CV's of the same buffer. rhLAMAN
25   is eluted with standard buffer and fractions containing activity are pooled and used for further purification.

     2.   *Macroprep, Ceramic hydroxyapatite type I or II.*
         Brief description: Equilibrate column (Gel volume = 20 ml) with 4-6 CV's of 10 mM
30   Sodium phosphate H 7.6 (Buffer A). Flow rate 2-5 ml/min. Buffer exchange sample from step 3 with TFF to Buffer A. Load sample from step 3 on column. Wash with 2-4 CV's of Buffer A. Elute with buffer A containing 100 mM NaCl. Collect peak containing rhLAMAN activity.

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Step 4: Intermediate step 2

1. *Macro prep, Ceramic hydroxyapatite type I or II, 40 um.*
   Brief description: see Step 3

5

2. *Source 15 Q – anioexchanger*
   Equilibrate column with 4 CV´s of 200 mM Tris-HCl pH 7.6. Change to 5 CV´s of 20 mM Tris-HCl pH 7.6 (standard buffer). Flow rate: 2- 5 ml/min. Load sample from step 3 (should be in standard buffer before application) onto column. Wash with 2-

10   4 CV´s of standard buffer. Apply a shallow gradient from 0 to 100% of 1 M NaCl in standard buffer (flow rate 2ml/min, gradient time 50 minutes). Collect fractions containing rhLAMAN activity.

3. *Source 15 S – cation exchanger*

15   Should run in acidic pH. Equilibration buffer = 20 mM Sodium Acetate pH 4.5. Elute with a NaCl (increasing salt concentration) or pH (increasing pH) gradient.

Step 5: Polishing step

20   1. *Macro prep, Ceramic hydroxyapatite type II, 40 um.*
   Description of parameters: see Step 3.

2. *Source 15 Q – anion exchanger*
   Description of parameters: see Step 4.

25

3. *Source 15 S – cation exchanger*
   Description of parameters: see Step 4.

4. *Affinity chromatography*

30

Step 6: Diafiltration / Formulation step
Tangential flow filtration (TFF) against a Pellicon Biomax polysulphone filter with 100 kDa cut off against 5-10 x Volumes of formulation buffer is performed. Two formulation buffers

35   are tested:

Formulation buffer 1.
Na_2HPO_4 3.10-3.50 mM

38

NaH$_2$PO$_4$ 0.4-0.6 mM

Glycine   25-30 mM

Mannitol   220-250 mM

Water for injection (WFI)

5

Formulation buffer 2.

Sodium Citrate   4.0-5.0 mM

Citric Acid   0.3-0.8 mM

Mannitol   200-250 mM

10 Tween 80   3.0-5.0 mM

Water for injection (WFI)

The pH and osmolality in both Formulation buffers are balanced to 7.5 ± 0.2 and 300 ± 50

mOsm/kg respectively. Final protein concentration is according to the specification (>5

15 mg/ml).

Step 7: Formulation, Filling and Freeze-drying

Formulation and dosage form

In the development of dosage form the stability of rhLAMAN is focused. The development

20 process starts with an aqueous solution and will, most likely, end up as a freeze-dried

product. Two different formulations are tested: Formulation buffer 1 and Formulation

buffer 6, see Step 6.

Both these formulations are known to stabilize proteins in aqueous solutions as well as in

25 freeze-dried powders. The pH and osmolality in both Formulation buffers will be balanced

to 7.5 ± 0.2 and 300 ± 50 mOsm/kg respectively. Final protein concentration should be

according to the specification and in the range 4-10 mg/ml.

A freeze-dried product of rhLAMAN will be produced at a production unit according to EU

30 GMP practice. The filling and freeze-drying will be performed in a room classified as Class

A. During production the filling zone is monitored with particle count and settle plates. The

personnel are regularly trained according to EU GMP and monitored after each production

with glove prints. The sterility of equipment and materials are secured by validated

sterilization procedures.

35

Filling

The bulk drug substance of rhLAMAN are aseptically filled in sterile type I glass vials.

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Freeze-drying

The vials are freeze-dried with freeze-drying cycles specifically developed for rhLAMAN in the two different formulations described above. Nitrogen gas is filled into the vials in the
5　end of the cycle and eventually closed with stoppers and capped. The batch is finally analyzed and released according to the specification.

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WO 02/099092   PCT/DK02/00388

45

INDICATIONS RELATING TO DEPOSITED MICROORGANISM
OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13bis)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description
on page __9__ , line __13-15__ .

B. IDENTIFICATION OF DEPOSIT   Further deposits are identified on an additional sheet ☐

Name of depositary institution

Deutsche Sammlung von Microorganismen und Zellkulturen GmbH

Address of depositary institution (including postal code and country)

Maschroderweg 1b
D-38124 Braunschweig
Federal Republic of Germany

Date of deposit	Accession Number
6 June 2002	DSM ACC2549

C. ADDITIONAL INDICATIONS (leave blank if not applicable)   This information is continued on an additional sheet ☐

As regards the respective Patent Offices of the respective designated states, the applicant requests that a
sample of the deposited microorganism only be made available to an expert nominated by the requester
until the date on which the patent is granted or the date on which the application has been refused or
withdrawn or is deemed to be withdrawn.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g. "Accession
Number of Deposit")

For receiving Office use only	For International Bureau use only
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Authorized officer	Authorized officer

SUBSTITUTE SHEET

Form PCT/RO/134 (July1998)

46

CLAIMS

1. A cell capable of producing recombinant human LAMAN (rhLAMAN), said cell comprising the 3066 basepair EcoRI - XbaI fragment of a human cDNA which codes for a human
5   LAMAN protein in which the position corresponding to position 186 of the full length hLAMAN protein is Aspartic acid (Asp, D).

2. A cell according to claim 1 comprising a DNA sequence which codes for the amino acid sequence shown in SEQ ID NO 12.
10

3. A cell according to claim 1 obtained by insertion of a DNA fragment comprising the 3066 basepair EcoRI - XbaI fragment of the DNA fragment having the sequence shown in SEQ ID NO 2.

15   4. A cell according to any of claims 1 - 3 obtained by use of the expression plasmid pLamanExp1 having SEQ ID NO. 2.

5. A cell according to any of claims 1 - 4 obtained by transfection of a non-human mammalian cell line.
20

6. A cell according to claim 5 obtained by transfection of chinese hamster ovary (CHO) cells.

7. A cell according to any of claims 1 - 6 obtained by the culture of the human LAMAN
25   production cell line CHO DG44.42 subclone 20L-31.62.5 which has been deposited at the DSMZ for the purposes of patent deposit according to the Budapest Treaty on 6 June 2002.

8. A method for the preparation of recombinant human LAMAN, the method comprising

30   a) introducing, into a suitable vector, a nucleic acid fragment comprising a DNA fragment which codes for the amino acid sequence shown SEQ ID NO 12;

b) transforming a cell with the vector obtained in step a);

35   c) culturing the transformed host cell under conditions facilitating expression of the nucleic acid sequence;

d) recovering the expression product from the culture.

47

9. A method according to claim 8 further comprising a fermentation step.

10. A method according to claim 8 or 9 further comprising a purification step.

5   11. A method according to any of claims 8-10, wherein the nucleic acid fragment comprises the 3066 basepair EcoRI - XbaI fragment of the DNA fragment shown in SEQ ID NO 2.

12. An expression plasmid pLamanExp1 having the sequence shown in SEQ ID NO. 2.

10

13. A rhLAMAN produced by the method of any of claims 8-11.

14. Use of the rhLAMAN according to claim 13 for the preparation of a medicament for the treatment of alfa mannosidosis.
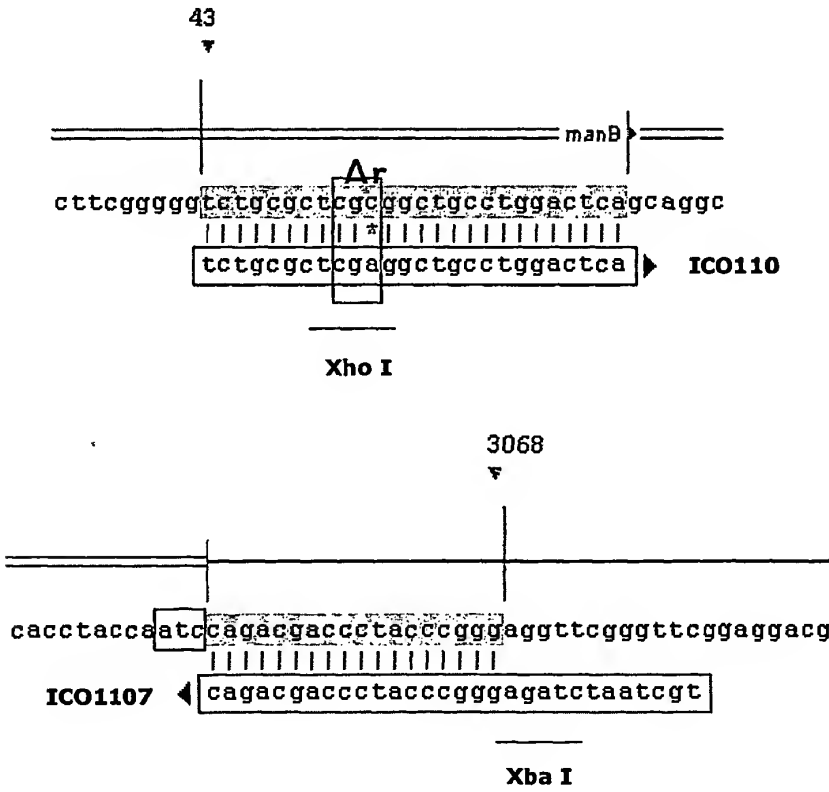
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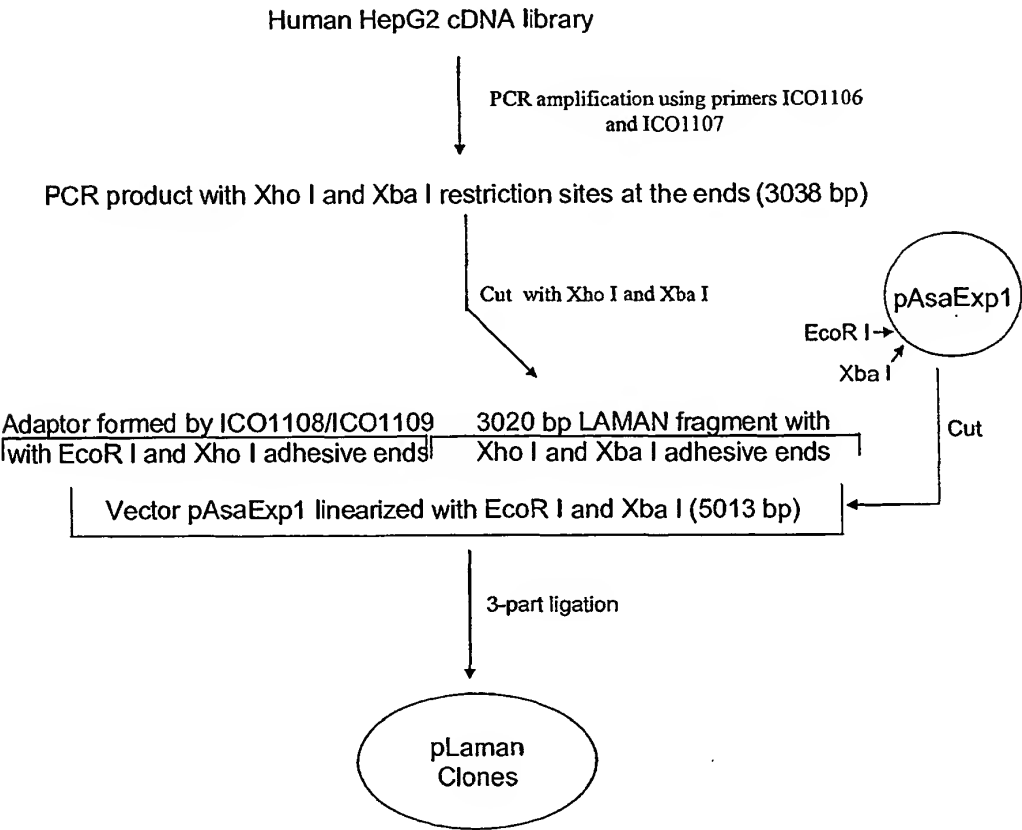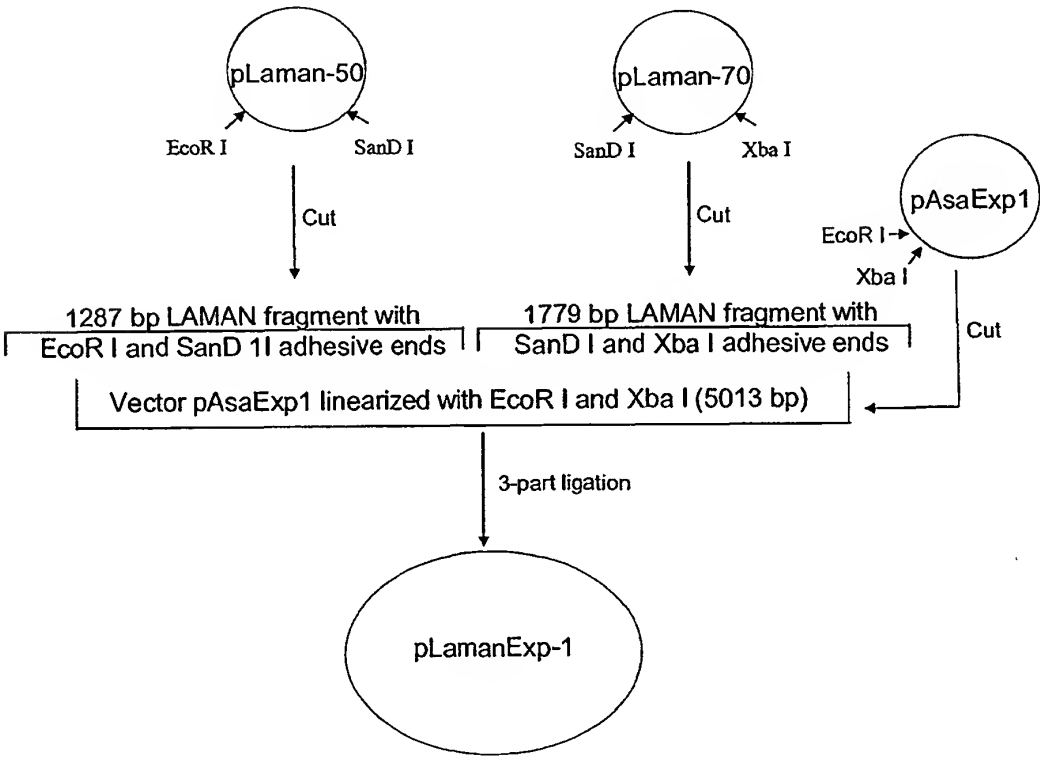
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Xho I

3068

⬇

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Xba I

Fig. 1

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STEP I

Human HepG2 cDNA library

PCR amplification using primers ICO1106
and ICO1107

PCR product with Xho I and Xba I restriction sites at the ends (3038 bp)

Cut with Xho I and Xba I

pAsaExp1

EcoR I →
Xba I

Cut

Adaptor formed by ICO1108/ICO1109   3020 bp LAMAN fragment with
with EcoR I and Xho I adhesive ends   Xho I and Xba I adhesive ends

Vector pAsaExp1 linearized with EcoR I and Xba I (5013 bp)

3-part ligation

pLaman
Clones

Fig. 2

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STEP II

Fig. 2 continued

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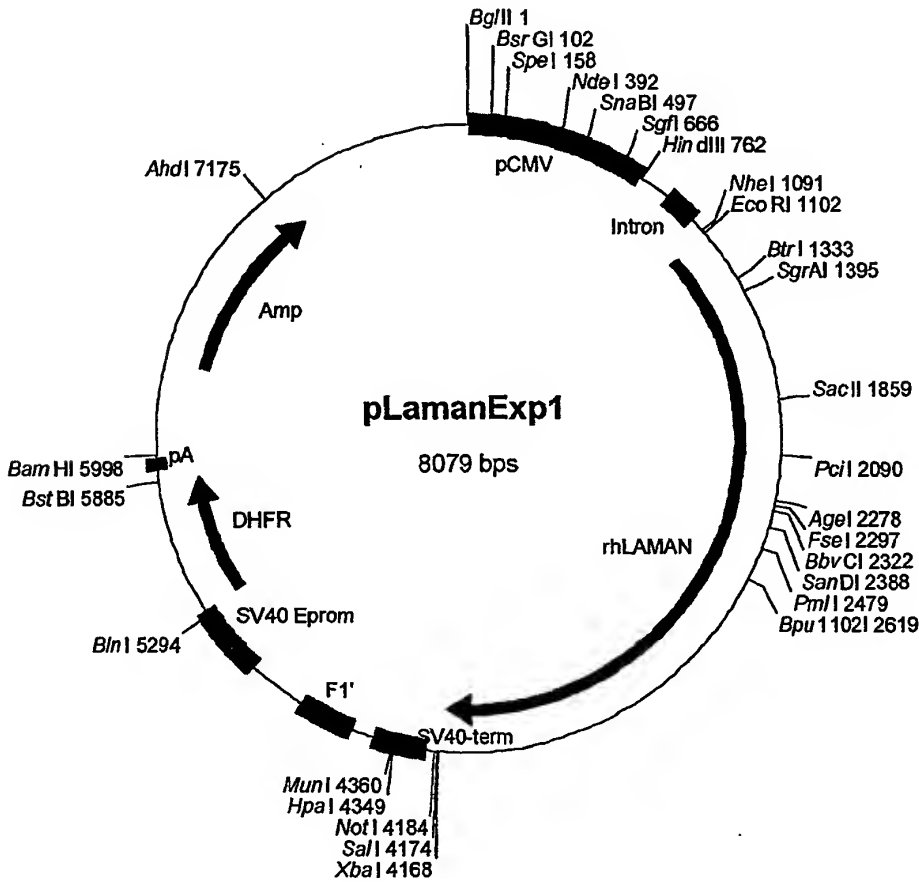
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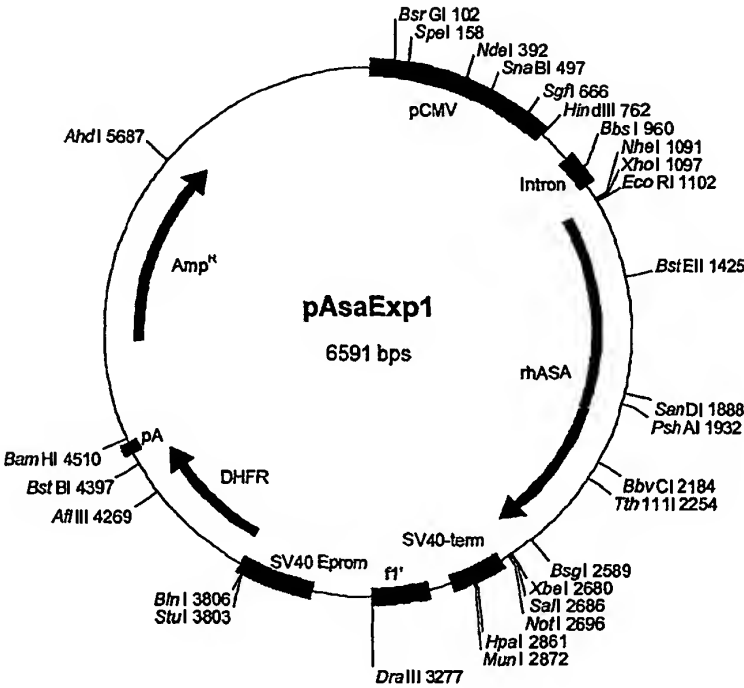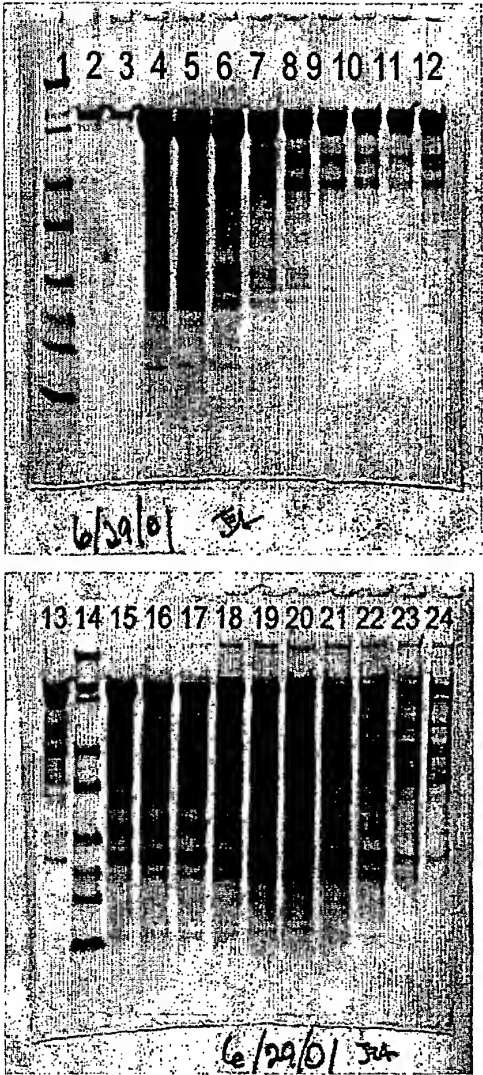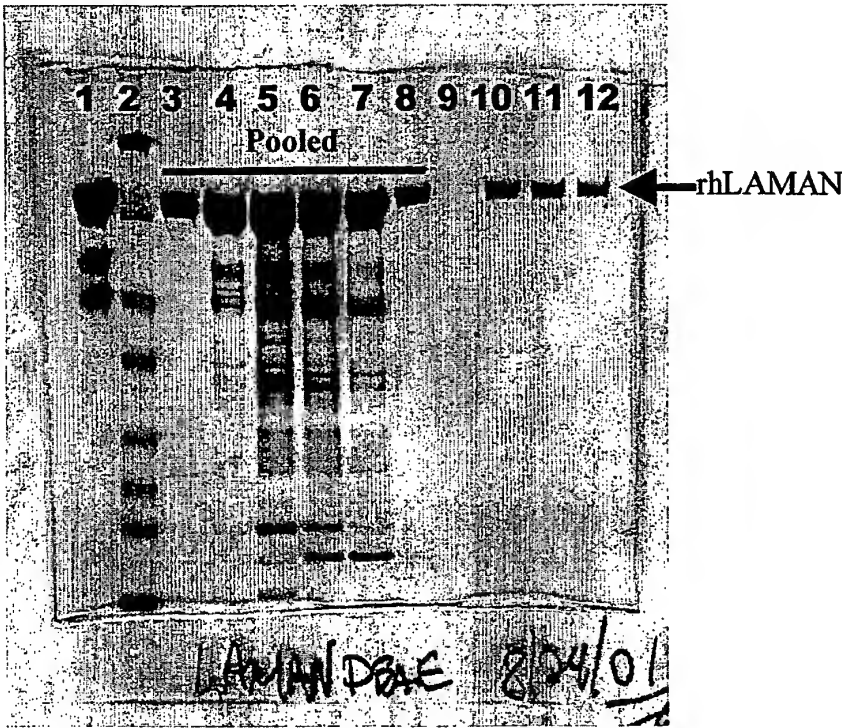
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Fig. 4

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Fig. 5

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Fig. 6

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Fig. 7

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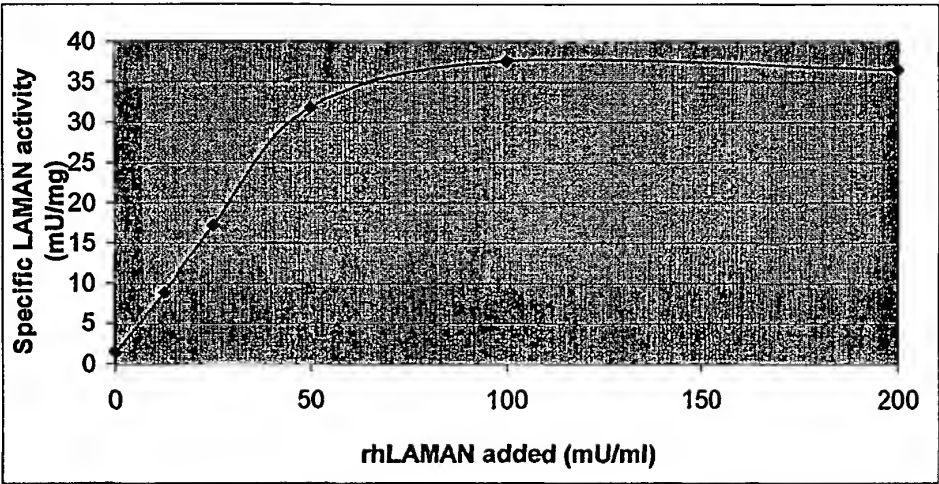
Fig. 8

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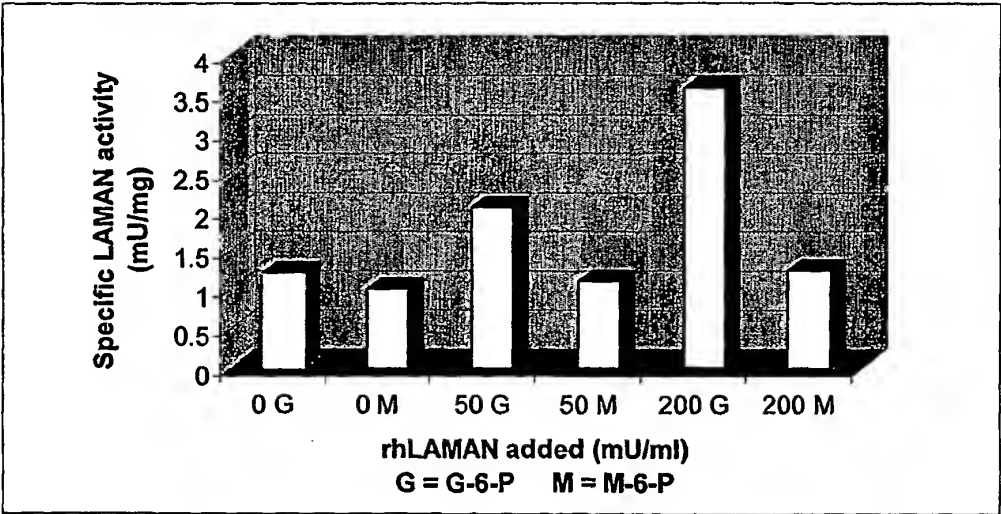<120> Production of recombinant human
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Ser Gly Val Cys Ala Arg Gly Cys Leu Asp Ser Ala Gly Pro Trp Thr
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atg tcc cgc gcc ctg cgg cca ccg ctc ccg cct ctc tgc ttt ttc ctt    1230
Met Ser Arg Ala Leu Arg Pro Pro Leu Pro Pro Leu Cys Phe Phe Leu
    25              30              35

ttg ttg ctg gcg gct gcc ggt gct cgg gcc ggg gga tac gag aca tgc    1278
Leu Leu Leu Ala Ala Ala Gly Ala Arg Ala Gly Gly Tyr Glu Thr Cys
40              45              50              55

ccc aca gtg cag ccg aac atg ctg aac gtg cac ctg ctg cct cac aca    1326
Pro Thr Val Gln Pro Asn Met Leu Asn Val His Leu Leu Pro His Thr
                60              65              70

cat gat gac gtg ggc tgg ctc aaa acc gtg gac cag tac ttt tat gga    1374
His Asp Asp Val Gly Trp Leu Lys Thr Val Asp Gln Tyr Phe Tyr Gly
            75              80              85

atc aag aat gac atc cag cac gcc ggt gtg cag tac atc ctg gac tcg    1422
Ile Lys Asn Asp Ile Gln His Ala Gly Val Gln Tyr Ile Leu Asp Ser
        90              95              100

gtc atc tct gcc ttg ctg gca gat ccc acc cgt cgc ttc att tac gtg    1470
Val Ile Ser Ala Leu Leu Ala Asp Pro Thr Arg Arg Phe Ile Tyr Val
    105             110             115

gag att gcc ttc ttc tcc cgt tgg tgg cac cag cag aca aat gcc aca    1518
Glu Ile Ala Phe Phe Ser Arg Trp Trp His Gln Gln Thr Asn Ala Thr
120             125             130             135

cag gaa gtc gtg cga gac ctt gtg cgc cag ggg cgc ctg gag ttc gcc    1566
Gln Glu Val Val Arg Asp Leu Val Arg Gln Gly Arg Leu Glu Phe Ala
                140             145             150

aat ggt ggc tgg gtg atg aac gat gag gca gcc acc cac tac ggt gcc    1614
Asn Gly Gly Trp Val Met Asn Asp Glu Ala Ala Thr His Tyr Gly Ala
                155             160             165

atc gtg gac cag atg aca ctt ggg ctg cgc ttt ctg gag gac aca ttt    1662
Ile Val Asp Gln Met Thr Leu Gly Leu Arg Phe Leu Glu Asp Thr Phe
            170             175             180

ggc aat gat ggg cga ccc cgt gtg gcc tgg cac att gac ccc ttc ggc    1710
Gly Asn Asp Gly Arg Pro Arg Val Ala Trp His Ile Asp Pro Phe Gly
        185             190             195

cac tct cgg gag cag gcc tcg ctg ttt gcg cag atg ggc ttc gac ggc    1758
His Ser Arg Glu Gln Ala Ser Leu Phe Ala Gln Met Gly Phe Asp Gly
200             205             210             215

ttc ttc ttt ggg cgc ctt gat tat caa gat aag tgg gta cgg atg cag    1806
Phe Phe Phe Gly Arg Leu Asp Tyr Gln Asp Lys Trp Val Arg Met Gln
                220             225             230
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aag ctg gag atg gag cag gtg tgg cgg gcc agc acc agc ctg aag ccc     1854
Lys Leu Glu Met Glu Gln Val Trp Arg Ala Ser Thr Ser Leu Lys Pro
            235             240             245

ccg acc gcg gac ctc ttc act ggt gtg ctt ccc aat ggt tac aac ccg     1902
Pro Thr Ala Asp Leu Phe Thr Gly Val Leu Pro Asn Gly Tyr Asn Pro
            250             255             260

cca agg aat ctg tgc tgg gat gtg ctg tgt gtc gat cag ccg ctg gtg     1950
Pro Arg Asn Leu Cys Trp Asp Val Leu Cys Val Asp Gln Pro Leu Val
            265             270             275

gag gac cct cgc agc ccc gag tac aac gcc aag gag ctg gtc gat tac     1998
Glu Asp Pro Arg Ser Pro Glu Tyr Asn Ala Lys Glu Leu Val Asp Tyr
280             285             290             295

ttc cta aat gtg gcc act gcc cag ggc cgg tat tac cgc acc aac cac     2046
Phe Leu Asn Val Ala Thr Ala Gln Gly Arg Tyr Tyr Arg Thr Asn His
            300             305             310

act gtg atg acc atg ggc tcg gac ttc caa tat gag aat gcc aac atg     2094
Thr Val Met Thr Met Gly Ser Asp Phe Gln Tyr Glu Asn Ala Asn Met
            315             320             325

tgg ttc aag aac ctt gac aag ctc atc cgg ctg gta aat gcg cag cag     2142
Trp Phe Lys Asn Leu Asp Lys Leu Ile Arg Leu Val Asn Ala Gln Gln
            330             335             340

gca aaa gga agc agt gtc cat gtt ctc tac tcc acc ccc gct tgt tac     2190
Ala Lys Gly Ser Ser Val His Val Leu Tyr Ser Thr Pro Ala Cys Tyr
            345             350             355

ctc tgg gag ctg aac aag gcc aac ctc acc tgg tca gtg aaa cat gac     2238
Leu Trp Glu Leu Asn Lys Ala Asn Leu Thr Trp Ser Val Lys His Asp
360             365             370             375

gac ttc ttc cct tac gcg gat ggc ccc cac cag ttc tgg acc ggt tac     2286
Asp Phe Phe Pro Tyr Ala Asp Gly Pro His Gln Phe Trp Thr Gly Tyr
            380             385             390

ttt tcc agt cgg ccg gcc ctc aaa cgc tac gag cgc ctc agc tac aac     2334
Phe Ser Ser Arg Pro Ala Leu Lys Arg Tyr Glu Arg Leu Ser Tyr Asn
            395             400             405

ttc ctg cag gtg tgc aac cag ctg gag gcg ctg gtg ggc ctg gcg gcc     2382
Phe Leu Gln Val Cys Asn Gln Leu Glu Ala Leu Val Gly Leu Ala Ala
            410             415             420

aac gtg gga ccc tat ggc tcc gga gac agt gca ccc ctc aat gag gcg     2430
Asn Val Gly Pro Tyr Gly Ser Gly Asp Ser Ala Pro Leu Asn Glu Ala
            425             430             435

atg gct gtg ctc cag cat cac gac gcc gtc agc ggc acc tcc cgc cag     2478
Met Ala Val Leu Gln His His Asp Ala Val Ser Gly Thr Ser Arg Gln
440             445             450             455

cac gtg gcc aac gac tac gcg cgc cag ctt gcg gca ggc tgg ggg cct     2526
His Val Ala Asn Asp Tyr Ala Arg Gln Leu Ala Ala Gly Trp Gly Pro
            460             465             470
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tgc gag gtt ctt ctg agc aac gcg ctg gcg cgg ctc aga ggc ttc aaa     2574
Cys Glu Val Leu Leu Ser Asn Ala Leu Ala Arg Leu Arg Gly Phe Lys
            475             480             485

gat cac ttc acc ttt tgc caa cag cta aac atc agc atc tgc ccg ctc     2622
Asp His Phe Thr Phe Cys Gln Gln Leu Asn Ile Ser Ile Cys Pro Leu
        490             495             500

agc cag acg gcg gcg cgc ttc cag gtc atc gtt tat aat ccc ctg ggg     2670
Ser Gln Thr Ala Ala Arg Phe Gln Val Ile Val Tyr Asn Pro Leu Gly
        505             510             515

cgg aag gtg aat tgg atg gta cgg ctg ccg gtc agc gaa ggc gtt ttc     2718
Arg Lys Val Asn Trp Met Val Arg Leu Pro Val Ser Glu Gly Val Phe
520             525             530             535

gtt gtg aag gac ccc aat ggc agg aca gtg ccc agc gat gtg gta ata     2766
Val Val Lys Asp Pro Asn Gly Arg Thr Val Pro Ser Asp Val Val Ile
            540             545             550

ttt ccc agc tca gac agc cag gcg cac cct ccg gag ctg ctg ttc tca     2814
Phe Pro Ser Ser Asp Ser Gln Ala His Pro Pro Glu Leu Leu Phe Ser
            555             560             565

gcc tca ctg ccc gcc ctg ggc ttc agc acc tat tca gta gcc cag gtg     2862
Ala Ser Leu Pro Ala Leu Gly Phe Ser Thr Tyr Ser Val Ala Gln Val
            570             575             580

cct cgc tgg aag ccc cag gcc cgc gca cca cag ccc atc ccc aga aga     2910
Pro Arg Trp Lys Pro Gln Ala Arg Ala Pro Gln Pro Ile Pro Arg Arg
        585             590             595

tcc tgg tcc cct gct tta acc atc gaa aat gag cac atc cgg gca acg     2958
Ser Trp Ser Pro Ala Leu Thr Ile Glu Asn Glu His Ile Arg Ala Thr
600             605             610             615

ttt gat cct gac aca ggg ctg ttg atg gag att atg aac atg aat cag     3006
Phe Asp Pro Asp Thr Gly Leu Leu Met Glu Ile Met Asn Met Asn Gln
            620             625             630

caa ctc ctg ctg cct gtt cgc cag acc ttc ttc tgg tac aac gcc agt     3054
Gln Leu Leu Leu Pro Val Arg Gln Thr Phe Phe Trp Tyr Asn Ala Ser
            635             640             645

ata ggt gac aac gaa agt gac cag gcc tca ggt gcc tac atc ttc aga     3102
Ile Gly Asp Asn Glu Ser Asp Gln Ala Ser Gly Ala Tyr Ile Phe Arg
            650             655             660

ccc aac caa cag aaa ccg ctg cct gtg agc cgc tgg gct cag atc cac     3150
Pro Asn Gln Gln Lys Pro Leu Pro Val Ser Arg Trp Ala Gln Ile His
        665             670             675

ctg gtg aag aca ccc ttg gtg cag gag gtg cac cag aac ttc tca gct     3198
Leu Val Lys Thr Pro Leu Val Gln Glu Val His Gln Asn Phe Ser Ala
680             685             690             695

tgg tgt tcc cag gtg gtt cgc ctg tac cca gga cag cgg cac ctg gag     3246
Trp Cys Ser Gln Val Val Arg Leu Tyr Pro Gly Gln Arg His Leu Glu
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cta gag tgg tcg gtg ggg ccg ata cct gtg ggc gac acc tgg ggg aag   3294
Leu Glu Trp Ser Val Gly Pro Ile Pro Val Gly Asp Thr Trp Gly Lys
            715                720                725

gag gtc atc agc cgt ttt gac aca ccg ctg gag aca aag gga cgc ttc   3342
Glu Val Ile Ser Arg Phe Asp Thr Pro Leu Glu Thr Lys Gly Arg Phe
            730                735                740

tac aca gac agc aat ggc cgg gag atc ctg gag agg agg cgg gat tat   3390
Tyr Thr Asp Ser Asn Gly Arg Glu Ile Leu Glu Arg Arg Arg Asp Tyr
            745                750                755

cga ccc acc tgg aaa ctg aac cag acg gag ccc gtg gca gga aac tac   3438
Arg Pro Thr Trp Lys Leu Asn Gln Thr Glu Pro Val Ala Gly Asn Tyr
760                765                770                775

tat cca gtc aac acc cgg att tac atc acg gat gga aac atg cag ctg   3486
Tyr Pro Val Asn Thr Arg Ile Tyr Ile Thr Asp Gly Asn Met Gln Leu
                   780                785                790

act gtg ctg act gac cgc tcc cag ggg ggc agc agc ctg aga gat ggc   3534
Thr Val Leu Thr Asp Arg Ser Gln Gly Gly Ser Ser Leu Arg Asp Gly
            795                800                805

tcg ctg gag ctc atg gtg cac cga agg ctg ctg aag gac gat gga cgc   3582
Ser Leu Glu Leu Met Val His Arg Arg Leu Leu Lys Asp Asp Gly Arg
            810                815                820

gga gta tcg gag cca cta atg gag aac ggg tcg ggg gcg tgg gtg cga   3630
Gly Val Ser Glu Pro Leu Met Glu Asn Gly Ser Gly Ala Trp Val Arg
            825                830                835

ggg cgc cac ctg gtg ctg ctg gac aca gcc cag gct gca gcc gcc gga   3678
Gly Arg His Leu Val Leu Leu Asp Thr Ala Gln Ala Ala Ala Ala Gly
840                845                850                855

cac cgg ctc ctg gcg gag cag gag gtc ctg gcc cct cag gtg gtg ctg   3726
His Arg Leu Leu Ala Glu Gln Glu Val Leu Ala Pro Gln Val Val Leu
                   860                865                870

gcc ccg ggt ggc ggc gcc gcc tac aat ctc ggg gct cct ccg cgc acg   3774
Ala Pro Gly Gly Gly Ala Ala Tyr Asn Leu Gly Ala Pro Pro Arg Thr
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cag ttc tca ggg ctg cgc agg gac ctg ccg ccc tcg gtg cac ctg ctc   3822
Gln Phe Ser Gly Leu Arg Arg Asp Leu Pro Pro Ser Val His Leu Leu
            890                895                900

acg ctg gcc agc tgg ggc ccc gaa atg gtg ctg ctg cgc ttg gag cac   3870
Thr Leu Ala Ser Trp Gly Pro Glu Met Val Leu Leu Arg Leu Glu His
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cag ttt gcc gta gga gag gat tcc gga cgt aac ctg agc gcc ccc gtt   3918
Gln Phe Ala Val Gly Glu Asp Ser Gly Arg Asn Leu Ser Ala Pro Val
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acc ttg aac ttg agg gac ctg ttc tcc acc ttc acc atc acc cgc ctg   3966
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Thr Leu Asn Leu Arg Asp Leu Phe Ser Thr Phe Thr Ile Thr Arg Leu
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cag gag acc acg ctg gtg gcc aac cag ctc cgc gag gca gcc tcc agg   4014
Gln Glu Thr Thr Leu Val Ala Asn Gln Leu Arg Glu Ala Ala Ser Arg
            955                 960                 965

ctc aag tgg aca aca aac aca ggc ccc aca ccc cac caa act ccg tac   4062
Leu Lys Trp Thr Thr Asn Thr Gly Pro Thr Pro His Gln Thr Pro Tyr
            970                 975                 980

cag ctg gac ccg gcc aac atc acg ctg gaa ccc atg gaa atc cgc act   4110
Gln Leu Asp Pro Ala Asn Ile Thr Leu Glu Pro Met Glu Ile Arg Thr
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Phe Leu Ala Ser Val Gln Trp Lys Glu Val Asp Gly  *
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            35                  40                  45
Ala Gly Gly Tyr Glu Thr Cys Pro Thr Val Gln Pro Asn Met Leu Asn
    50                  55                  60
Val His Leu Leu Pro His Thr His Asp Asp Val Gly Trp Leu Lys Thr
65                  70                  75                  80
Val Asp Gln Tyr Phe Tyr Gly Ile Lys Asn Asp Ile Gln His Ala Gly
                85                  90                  95
Val Gln Tyr Ile Leu Asp Ser Val Ile Ser Ala Leu Leu Ala Asp Pro
                100                 105                 110
Thr Arg Arg Phe Ile Tyr Val Glu Ile Ala Phe Phe Ser Arg Trp Trp
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His Gln Gln Thr Asn Ala Thr Gln Glu Val Val Arg Asp Leu Val Arg
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Gln Gly Arg Leu Glu Phe Ala Asn Gly Gly Trp Val Met Asn Asp Glu
145                 150                 155                 160
Ala Ala Thr His Tyr Gly Ala Ile Val Asp Gln Met Thr Leu Gly Leu
                165                 170                 175
Arg Phe Leu Glu Asp Thr Phe Gly Asn Asp Gly Arg Pro Arg Val Ala
            180                 185                 190
Trp His Ile Asp Pro Phe Gly His Ser Arg Glu Gln Ala Ser Leu Phe
            195                 200                 205
Ala Gln Met Gly Phe Asp Gly Phe Phe Phe Gly Arg Leu Asp Tyr Gln
        210                 215                 220
Asp Lys Trp Val Arg Met Gln Lys Leu Glu Met Glu Gln Val Trp Arg
225                 230                 235                 240
Ala Ser Thr Ser Leu Lys Pro Pro Thr Ala Asp Leu Phe Thr Gly Val
                245                 250                 255
Leu Pro Asn Gly Tyr Asn Pro Pro Arg Asn Leu Cys Trp Asp Val Leu
                260                 265                 270
Cys Val Asp Gln Pro Leu Val Glu Asp Pro Arg Ser Pro Glu Tyr Asn
        275                 280                 285
Ala Lys Glu Leu Val Asp Tyr Phe Leu Asn Val Ala Thr Ala Gln Gly
        290                 295                 300
Arg Tyr Tyr Arg Thr Asn His Thr Val Met Thr Met Gly Ser Asp Phe
305                 310                 315                 320
Gln Tyr Glu Asn Ala Asn Met Trp Phe Lys Asn Leu Asp Lys Leu Ile
                325                 330                 335
Arg Leu Val Asn Ala Gln Gln Ala Lys Gly Ser Ser Val His Val Leu
            340                 345                 350
Tyr Ser Thr Pro Ala Cys Tyr Leu Trp Glu Leu Asn Lys Ala Asn Leu
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            355                   360                   365
Thr Trp Ser Val Lys His Asp Asp Phe Phe Pro Tyr Ala Asp Gly Pro
    370                   375                   380
His Gln Phe Trp Thr Gly Tyr Phe Ser Ser Arg Pro Ala Leu Lys Arg
385                   390                   395                   400
Tyr Glu Arg Leu Ser Tyr Asn Phe Leu Gln Val Cys Asn Gln Leu Glu
                405                   410                   415
Ala Leu Val Gly Leu Ala Ala Asn Val Gly Pro Tyr Gly Ser Gly Asp
                420                   425                   430
Ser Ala Pro Leu Asn Glu Ala Met Ala Val Leu Gln His Asp Ala
            435                   440                   445
Val Ser Gly Thr Ser Arg Gln His Val Ala Asn Asp Tyr Ala Arg Gln
    450                   455                   460
Leu Ala Ala Gly Trp Gly Pro Cys Glu Val Leu Leu Ser Asn Ala Leu
465                   470                   475                   480
Ala Arg Leu Arg Gly Phe Lys Asp His Phe Thr Phe Cys Gln Gln Leu
                485                   490                   495
Asn Ile Ser Ile Cys Pro Leu Ser Gln Thr Ala Ala Arg Phe Gln Val
                500                   505                   510
Ile Val Tyr Asn Pro Leu Gly Arg Lys Val Asn Trp Met Val Arg Leu
            515                   520                   525
Pro Val Ser Glu Gly Val Phe Val Val Lys Asp Pro Asn Gly Arg Thr
    530                   535                   540
Val Pro Ser Asp Val Val Ile Phe Pro Ser Ser Asp Ser Gln Ala His
545                   550                   555                   560
Pro Pro Glu Leu Leu Phe Ser Ala Ser Leu Pro Ala Leu Gly Phe Ser
                565                   570                   575
Thr Tyr Ser Val Ala Gln Val Pro Arg Trp Lys Pro Gln Ala Arg Ala
                580                   585                   590
Pro Gln Pro Ile Pro Arg Arg Ser Trp Ser Pro Ala Leu Thr Ile Glu
            595                   600                   605
Asn Glu His Ile Arg Ala Thr Phe Asp Pro Asp Thr Gly Leu Leu Met
    610                   615                   620
Glu Ile Met Asn Met Asn Gln Gln Leu Leu Leu Pro Val Arg Gln Thr
625                   630                   635                   640
Phe Phe Trp Tyr Asn Ala Ser Ile Gly Asp Asn Glu Ser Asp Gln Ala
                645                   650                   655
Ser Gly Ala Tyr Ile Phe Arg Pro Asn Gln Gln Lys Pro Leu Pro Val
                660                   665                   670
Ser Arg Trp Ala Gln Ile His Leu Val Lys Thr Pro Leu Val Gln Glu
            675                   680                   685
Val His Gln Asn Phe Ser Ala Trp Cys Ser Gln Val Val Arg Leu Tyr
    690                   695                   700
Pro Gly Gln Arg His Leu Glu Leu Glu Trp Ser Val Gly Pro Ile Pro
705                   710                   715                   720
Val Gly Asp Thr Trp Gly Lys Glu Val Ile Ser Arg Phe Asp Thr Pro
                725                   730                   735
Leu Glu Thr Lys Gly Arg Phe Tyr Thr Asp Ser Asn Gly Arg Glu Ile
                740                   745                   750
Leu Glu Arg Arg Arg Asp Tyr Arg Pro Thr Trp Lys Leu Asn Gln Thr
            755                   760                   765
Glu Pro Val Ala Gly Asn Tyr Tyr Pro Val Asn Thr Arg Ile Tyr Ile
    770                   775                   780
Thr Asp Gly Asn Met Gln Leu Thr Val Leu Thr Asp Arg Ser Gln Gly
785                   790                   795                   800
Gly Ser Ser Leu Arg Asp Gly Ser Leu Glu Leu Met Val His Arg Arg
                805                   810                   815
Leu Leu Lys Asp Asp Gly Arg Gly Val Ser Glu Pro Leu Met Glu Asn
                820                   825                   830
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Gly Ser Gly Ala Trp Val Arg Gly Arg His Leu Val Leu Leu Asp Thr
    835                 840             845
Ala Gln Ala Ala Ala Ala Gly His Arg Leu Leu Ala Glu Gln Glu Val
    850             855             860
Leu Ala Pro Gln Val Val Leu Ala Pro Gly Gly Gly Ala Ala Tyr Asn
865             870             875                 880
Leu Gly Ala Pro Pro Arg Thr Gln Phe Ser Gly Leu Arg Arg Asp Leu
            885             890                 895
Pro Pro Ser Val His Leu Leu Thr Leu Ala Ser Trp Gly Pro Glu Met
            900             905             910
Val Leu Leu Arg Leu Glu His Gln Phe Ala Val Gly Glu Asp Ser Gly
            915             920             925
Arg Asn Leu Ser Ala Pro Val Thr Leu Asn Leu Arg Asp Leu Phe Ser
    930             935             940
Thr Phe Thr Ile Thr Arg Leu Gln Glu Thr Thr Leu Val Ala Asn Gln
945             950             955                 960
Leu Arg Glu Ala Ala Ser Arg Leu Lys Trp Thr Thr Asn Thr Gly Pro
            965             970                 975
Thr Pro His Gln Thr Pro Tyr Gln Leu Asp Pro Ala Asn Ile Thr Leu
            980             985             990
Glu Pro Met Glu Ile Arg Thr Phe Leu Ala Ser Val Gln Trp Lys Glu
    995             1000            1005
Val Asp Gly
    1010
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